New Genes that Act Downstream of a FoxO Transcription Factor to Regulate Diapause and Longevity

by

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Dedication

To all of you who gave me the opportunity to do something worthwhile.

Acknowledgements

The more I learn about the life-sciences, the more I am always amazed by the cascade of changes that can unfold from a single chance event: changing one base-pair on one strand of DNA can cause a life-long illness or make an organism that can maintain good health for much longer than its siblings. Similarly, it's amazing how much our lives are shaped by a history of chance interactions with other people. I would like to thank a few of the wonderful people who have made this dissertation possible.

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Preface

Why must we age and die? This might be the oldest question humans have asked, it is certainly one of the most recurrent. One might think that a century after the discovery of genetics set off rapid advancements in biology that have led to atomic level understanding of many mechanisms underlying development, physiology, and pathology, that it would have a satisfying answer. It doesn't.

One way inside the mystery of aging has been provided by the discovery that a single mutation which reduces the activity of an insulin receptor family member can dramatically extend longevity and delay all visible signs of aging in the simple nematode *C. elegans*. The delay in aging requires increased activity of another gene, *daf-16*, that encodes a FoxO family transcription factor. DAF-16 is the switch that controls a genetic program; it turns up the levels of many other gene products and turns down the levels of others. If we can understand this downstream program, maybe can understand why aging is delayed when DAF-16 is on, and from there we can get a real answer as to what causes aging in the first place. If we can find good ways to regulate activity of *daf-16*-like genes in humans, we might be able to delay or prevent multiple age-related pathologies and increase the number of years we can spend in good health.

This dissertation is comprised of two parallel strategies. Chapters One and Two will discuss a strategy that was designed to identify novel mechanisms that regulate DAF-16 activity. Chapters Three and Four will discuss a strategy that was designed to find genes that act downstream of DAF-16 to regulate aging. Thus, Chapters One and Three will

review context and background material relevant to each approach, while Chapters Two and Four will discuss experimental work.

Design and conception of most experiments was performed independently under the guidance of Dr. Patrick Hu, with additional guidance from Dr. Scott Leiser. Chapter Two includes a forward genetic screen that was performed by Dr. Kathleen Dumas, and some initial mapping experiments that were carried out by Dr. Albert Chen. Chapter Four contains transcriptome profiling and quantitative-pcr experiments that were initially carried out by Dr. Albert Chen, with support from members of the University of Michigan DNA Sequencing Core and Bioinformatics Core, including Ana Rodrigues, Richard McEachin, and Manjusha Pande. Dr. Shohei Mitani (Tokyo Women's Medical University) created some mutant strains that were used in Chapter 4. Testing and outcrossing of candidate mutations in Chapter 4 was a collaborative effort that included Dr. Albert Chen, Ian Waters, Chun-Fang Guo, Breane Budaitis, and myself. Some confocal microscope images used in Chapter 4 were acquired with training and assistance from Dr. Colin Delaney

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Abstract

Age related diseases are leading causes of mortality worldwide. Environmental interventions such as dietary restriction can both extend lifespan and delay or ameliorate age related pathologies. Dietary restriction and other environmental stresses activate conserved stress response mechanisms that can delay many aspects of biological aging. Understanding the regulation of these stress responses and characterizing their biological mechanisms could enable the design of therapeutics to treat or prevent age-related diseases by directly targeting the aging process.

Reductions in the activity of the conserved insulin and insulin-like growth factor signaling pathway (IIS) extend longevity across taxa. IIS antagonizes FoxO transcription factors, and increased FoxO activity promotes increased longevity in multiple organisms. Polymorphisms in human FoxO genes are associated with longevity, and FoxO transcription factors likely modulate various age-related diseases. Thus, an improved understanding of the mechanisms regulating FoxO activity and the mechanisms through which FoxO activity increases lifespan may be relevant to human health.

The short-lived nematode *Caenorhabditis elegans* provides a useful model for studying FoxO regulation and aging. In *C. elegans,* the sole FoxO family member DAF-16 is inhibited by signals from an insulin-like growth factor receptor (IGFr) family member, DAF-2, and by signals from the germline. Disinhibition of DAF-16/FoxO activity during development causes entry into a larval diapause called dauer. Increasing DAF-16/FoxO

activity during adulthood via loss of function mutations in *daf-2*/IGFr or ablation of the germline dramatically increases lifespan. Thus, the dauer diapause represents a valuable system for understanding how DAF-16/FoxO activity is regulated, while studies of adult longevity can be used to understand how DAF-16/FoxO activation increases lifespan.

Reductions in IIS increase DAF-16/FoxO activity in part by causing it to be localized to the nucleus, where it can alter transcription of downstream target genes. Our group has discovered the EAK pathway, which inhibits nuclear DAF-16/FoxO activity. Mutations in *eak* genes enhance the constitutive dauer arrest phenotypes caused by mutation of the conserved gene *akt-1*. To identify downstream components of the EAK pathway we performed a forward genetic screen for suppression of dauer entry in *eak-7;akt-1* mutants. This screen unexpectedly identified a novel allele of *lin-14*, which encodes a transcription factor that acts to ensure the proper sequence of developmental events. This *lin-14* allele [*lin-14(dp69)*] functions along with *lin-14* RNAi to suppress dauer entry in both DAF-16/FoxO-dependent and DAF-16/FoxO-independent contexts, suggesting that LIN-14 acts downstream of, or in parallel to, DAF-16/FoxO to regulate dauer entry.

Two groups of transcriptional isoforms of DAF-16/FoxO, DAF-16A and DAF-16F, are required for increased lifespan. To identify downstream mechanisms by which DAF-16/FoxO activation increases longevity, we used transcriptome profiling to identify transcriptional events that are influenced by DAF-16A and F in both *daf-2*/IGFrmutants and mutants lacking a germline. We discovered that CEST-1, a previously unstudied member of the carboxylesterase family, is required for full lifespan extension in *daf-2*/IGFr mutants. CEST-1 expression is induced in *daf-2*/IGFr mutants, and CEST-1

localizes to the luminal membrane of the intestine. A conserved catalytic residue in the luminal CEST-1 domain is required for it to promote longevity, and CEST-1 overexpression is sufficient to increase lifespan. This evidence suggests that CEST-1 may eliminate a toxic metabolite in the gut or improve digestion of beneficial dietary components. Mammalian carboxylesterase family members are key regulators of metabolism, suggesting that carboxylesterase activity could regulate health and longevity in a conserved manner.

Chapter 1 - Mechanisms governing dauer formation in *C. elegans*

Introduction to *C. elegans* as a model for developmental plasticity

A central goal of the biological sciences is to understand, at a molecular level, the mechanisms which allow a single cell to develop into a complex adult organism composed of many interacting cells with identical genomes but diverse physiology and function. Over the last fifty years, studies of the simple nematode *Caenorhabditis elegans* have served as a central tool for elucidating conserved mechanisms underlying development.

C. elegans is a non-parasitic nematode which can be isolated from compost or rotting fruit samples around the world (Frézal & Félix, 2015). Adults are about a millimeter long and can be easily cultured in the lab on supplemented agar plates with an *E. coli* food source. *C. elegans* is transparent and develops through an invariant series of cell divisions into an adult with a fixed number and variety of somatic cells, permitting identification of genetic mutants which affect development. The nematode has two sexes, a hermaphrodite and a male, making it an ideal genetic system in which to either maintain genetically homogenous populations or perform genetic crosses for epistasis analysis (Brenner, 1974; Stiernagle, 2006). Genetic analysis in *C. elegans* has identified,

or improved our understanding of, numerous signal transduction mechanisms which are conserved in vertebrates.

A key phenomenon complicating development is plasticity-the ability of many organisms to alter their physiology and development in response to environmental signals (Fielenbach & Antebi, 2008). Along with other species of nematodes, *C. elegans* displays a dramatic form of developmental plasticity in its ability to stop development and undergo a lengthy arrest, or diapause, in an alternative developmental stage called the dauer larva. Under ideal conditions (culture at 15-25°C at low population density, with a constant food supply) *C. elegans* develops from hatching to adulthood in two to three days (depending on temperature) and passes through four distinct larval molts (L1-L4). Under stressful environmental conditions *C. elegans* can instead enter an alternative, extended, L2 larval phase called L2d-if adverse environmental conditions persist, L2d animals will progress to an alternative L3 larval stage called the dauer larva (Cassada & Russell, 1975; Golden & Riddle, 1984; Hu, 2007).

Characteristics of the dauer larval diapause

Dauer larvae are easily differentiated from developing larvae under a dissecting microscope. Dauer larvae are radially constricted, non-feeding, and do not move unless touched (Cassada & Russell, 1975). Their oral orifices are covered with a plug, and they have a specialized cuticle: these characteristics combine to render them resistant to many forms of environmental stress (Riddle et al., 1981). Dauer larvae can survive treatment with detergents (1% SDS), which enables them to be isolated easily from populations of non-dauer animals and facilitates genetic screens for mutants with increased tendency to

undergo the dauer life history (Cassada & Russell, 1975). Animals can persist as dauer larvae for months (many times the lifespan of well-fed adults) and then resume development to become adults with normal fertility and lifespan, leading to descriptions of the dauer larva as a "non-aging" state (Klass & Hirsh, 1976). Many other nematode species, including key human, animal, and plant parasites, undergo a dauer-like life-stage, which is often both the infective stage and an obligate part of development (Riddle et al., 1981). Thus, understanding regulation of the dauer life history of *C. elegans* may also lead to insights in combating parasitic nematode infections.

The developmental decision to undergo the dauer life history (frequently termed the "dauer decision") is influenced by sensory perception of three environmental parameters; food availability, temperature, and population density. *C. elegans* produces a soluble combination of ascarosides, frequently termed "dauer pheromone"; these ascarosides serve as a readout of population density (Butcher et al., 2007; Golden & Riddle, 1982; Jeong et al., 2005). Exogenous dauer pheromone can both induce dauer arrest in the absence of environmental stress and prevent dauer larvae from resuming normal development in replete conditions (Butcher et al., 2007; Golden & Riddle, 1982; Jeong et al., 2005). A soluble food signal derived from bacteria and yeast causes dauer larvae to resume reproductive development (Golden & Riddle, 1982). When nematodes are cultured on higher concentrations of bacterial food, higher doses of crude extracts containing pheromone are required to cause dauer arrest, suggesting that animals integrate information about population density and food availability to make the dauer decision (Golden & Riddle, 1984). Temperatures above 27°C cause dauer arrest in wild-type larvae, and increased temperature enhances dauer arrest in larvae treated with

pheromone, suggesting that animals also integrate information about temperature and population density to make the dauer decision (Golden & Riddle, 1984).

Animals exposed to dauer inducing-pheromone undergo a prolonged L2 larval stage called the L2d. At pheromone levels that cause dauer entry in only a fraction of the population, all animals enter the L2d larval stage, which can be followed by either dauer entry or reproductive development, demonstrating that L2d larvae are not fully committed to undergo dauer development (Golden & Riddle, 1984). Experiments in which animals undergoing development in synchronized populations are shifted from non-dauer inducing conditions to dauer-inducing conditions, or vice versa, have been used to determine at what developmental stage environmental signals act to control dauer arrest. For example, shifting animals from plates without pheromone to plates with pheromone at the L1 larval stage can induce dauer arrest; whereas animals shifted to pheromone containing plates during the early L2 stage do not enter dauer (Golden & Riddle, 1984). In contrast, shifting animals from pheromone to non-pheromone containing plates can prevent dauer arrest in most animals until the mid L2d larval stage (Golden & Riddle, 1984). These data suggest that the dauer decision is in fact composed of two distinct developmental periods during which environmental information is integrated before committing to dauer life history. During the L1 larval stage, perception of environmental stresses, including pheromone, low food, and temperature induce commitment to undergo the protracted L2d larval stage rather than committing to reproductive development through L2. During the late L2d larval stage information about changes in environmental conditions is integrated to either commit to the dauer life history or undergo reproductive development. Signal transduction pathways that regulate

the dauer decision may be involved in either or both developmental events, and the need to integrate multiple sensory inputs at two life stages to commit to the dauer life history may explain the surprising complexity of signal transduction pathways governing commitment to the dauer life history.

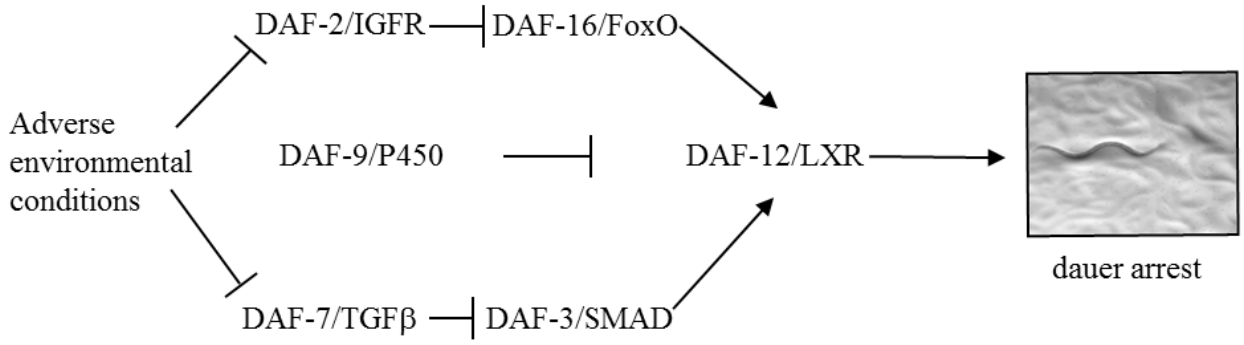
Identifying mutations that control the decision to undergo dauer diapause

Forward genetic screens have been conducted to identify mutations in genes that control the decision to undergo the dauer life-history. These screens have identified two primary classes of mutants. Mutants that are dauer formation constitutive (abbreviated Daf-c) enter dauer under conditions in which wild-type animals will undergo reproductive development. Screens for dauer constitutive mutants have primarily identified mutants that enter dauer at 25°C, or 27°C in synchronized cultures under non-crowded, fed conditions (Ailion & Thomas, 2003; Gottlieb & Ruvkun, 1994; Malone & Thomas, 1994; Riddle et al., 1981; Vowels & Thomas, 1992; Vowels & Thomas, 1994). A second class of mutants are dauer formation defective (Daf-d), meaning that they fail to enter dauer under dauer inducing conditions. Dauer formation defective mutants have been isolated by screening for mutations that block constitutive dauer formation in Daf-c strains (Ailion & Thomas, 2003; Gottlieb & Ruvkun, 1994; Inoue & Thomas, 2000a; Malone & Thomas, 1994; Riddle et al., 1981; Vowels & Thomas, 1992; Vowels & Thomas, 1994). The genetic epistatic relationships of these mutants have been extensively studied, and most dauer arrest mutants arising from initial screens have been mapped and the affected genes have been cloned. The dauer decision is regulated by genes that affect the sensory perception of dauer pheromone. Components of the insulin and TGF-β signaling

pathways are regulated, in part, by perception of dauer pheromone, and influence the dauer decision. Lastly, components of a steroid signaling pathway regulating the activity of the nuclear hormone receptor encoded by *daf-12*, regulated the dauer decision downstream of both TGF-β and insulin signaling (Hu, 2007).

Figure 1.1. Schematic of dauer regulation

Figure 1.1. Simplified schematic of the dauer regulatory network. In stressful environments signals from insulin, TGF-β, and steroid hormone biosynthesis pathways, converge on the nuclear hormone receptor DAF-12 to promote dauer entry



Perception of environmental stress informs the dauer decision via a guanylyl cyclase signaling pathway

The neural circuits that integrate information about population density, temperature, and food availability to commit to either the reproductive or dauer life history are incompletely understood. However, the genes that control dauer pheromone production, and some of the initiating events leading from pheromone perception to dauer formation have been discovered.

Three derivatives of the dideoxy sugar ascarylose, which differ only in their fatty acid side chains, have been isolated from dauer pheromone by purifying for dauer inducing or dauer recovery inhibiting activity (Butcher et al., 2007; Jeong et al., 2005). Organic extracts and culture medium of two mutants, *daf-22* and *dhs-28,* have reduced dauer inducing activity (Butcher et al., 2009). *daf-22* encodes a predicted thiolase with possible roles in fatty acid β-oxidation and synthesis of bile-acid like molecules, while *dhs-28* encodes a predicted dehydrogenase (Butcher et al., 2009). Analysis of the molecular composition of dauer pheromone extracts from these mutants is consistent with them acting in synthesis of the fatty acid side chains of dauer promoting ascarosides (Butcher et al., 2009).

Perception of pheromone is thought to primarily occur in two sensory organs called amphids which contain eight neurons which have cilia that are exposed to the external environment via a pore in the cuticle (Albert et al., 1981; Bargmann & Horvitz, 1991; Schackwitz et al., 1996). Like most somatic cells in *C. elegans*, the amphid neurons are individually named with a three-letter code. Ablations of the ADF, ASI, and ASG,

neurons cause dauer constitutive phenotypes, indicating that these neurons prevent dauer development under replete conditions, while ablation of the ASJ neuron prevents recovery from the dauer stage, suggesting that the ASJ is necessary for perception of improving environmental conditions to mediate recovery from dauer (Bargmann & Horvitz, 1991). Two mutants, *daf-6* and *daf-10*, were isolated as defective in dauer formation and have defects in the amphid pore (*daf-6*) or in sensory cilia (*daf-10*) that likely prevent sensory cilia from coming into contact with the external environment, suggesting that these mutants are defective in perception of dauer pheromone (Patrice et al., 2004). A third sensory mutant, *daf-19*, is dauer constitutive, and causes all neurons to completely lack cilia; however, this phenotype is unlikely to be specific to dauer pheromone perception since it affects many types of neurons (Swoboda et al., 2000).

Loss of function mutants in a transmembrane guanylyl cyclase, *daf-11*, that is expressed in chemosensory neurons (ASI, ASJ, ASK, AWB, and AWC) are Daf-c, as are mutations in two components of a cGMP gated ion channel (*tax-2* and *tax-4*) whose expression patterns overlap with that of DAF-11 (Birnby et al., 2000; Coburn & Bargmann, 1996; Coburn et al., 1998; Komatsu et al., 1996; Thomas et al., 1993). Treatment with 8-bromo cGMP rescues the dauer constitutive phenotype of *daf-11*, but not *tax-4* or other most other dauer constitutive mutants, suggesting that the predicted DAF-11 guanylyl cyclase activity acts upstream of, or in parallel to other dauer formation mutants (Birnby et al., 2000). Furthermore, the Daf-c phenotype of *daf-11* mutants is suppressed by *daf-6* and *daf-10* amphid neuron mutants (Starich et al., 1995; Vowels & Thomas, 1992). These data suggest that DAF-11 activation in chemosensory neurons is one of the earliest

events in a signaling pathway that inhibits dauer arrest under non-dauer inducing conditions.

Exposure to dauer pheromone reduces expression of two other dauer inhibiting genes, *daf-7* and *daf-28* (Li et al., 2003; Schackwitz et al., 1996). *daf-7* encodes a TGF-β family ligand; a DAF-7::GFP transgene is expressed in ASI and downregulated by exposure to dauer pheromone (Ren et al., 1996; Schackwitz et al., 1996). *daf-28* encodes an insulin-like peptide that is expressed in the ASI and ASJ and downregulated by dauer pheromone and in *daf-11* mutants (Li et al., 2003). This data suggests that DAF-11 acts upstream of both the insulin and TGF-β pathways to control dauer arrest.

Regulation of the dauer decision by a transforming-growth-factor-beta pathway

The DAF-7/TGF-β signaling network was originally defined by five Daf-c genes (*daf-1, 4, 7, 8,* and *14*) and two Daf-d genes (*daf-3* and *daf-5*) (Patterson & Padgett, 2000). The Transforming Growth-Factor-Beta (TGF-β) family ligand, encoded by *daf-7*, is inferred by homology to bind to a heteromeric cell surface receptor (encoded by *daf-1* and *daf-4*); based on homology to mammalian TGF-β pathway components DAF-1 and DAF-4 are thought to activate SMAD transcription factors (*daf-3*, *daf-8*, and *daf-14*) (Estevez et al., 1993; Inoue & Thomas, 2000b; Patterson et al., 1997; Shi & Massagué, 2003). The dauer constitutive phenotypes of *daf-8* and *daf-14* suggest that these genes are required for TGF-β pathway activation by DAF-7/ TGF-β to promote reproductive development, which is consistent with the known roles of mammalian SMAD transcription factors as positive effectors of TGF-β signaling (Inoue & Thomas, 2000b; Shi & Massagué, 2003).

In contrast, *daf-3*, whose mutation suppresses the dauer constitutive phenotypes of *daf-1*, *4*, *7*, *8*, and *14*, encodes a SMAD transcription factor that appears to be inhibited by DAF-7/ TGF-β and to promote entry into the dauer larval stage (Patterson et al., 1997). *daf-5* encodes a Sno/Ski oncoprotein that binds to *daf-3* and appears to be required for its dauer inducing activity (da Graca et al., 2004b; Tewari et al., 2004).

DAF-7::GFP constructs driven by the endogenous *daf-7* promoter can be expressed in the amphid chemosensory neurons including ASI and ASJ (Meisel et al., 2014; Ren et al., 1996; Schackwitz et al., 1996). DAF-7::GFP expression is increased by food and reduced by dauer pheromone and increased temperature, suggesting that DAF-7 levels influence dauer arrest in response to the environment (Ren et al., 1996; Schackwitz et al., 1996). Other components of the TGF-β pathway are broadly expressed (Da Graca et al., 2004a; Gunther et al., 2000; Inoue & Thomas, 2000b; Patterson et al., 1997; Tewari et al., 2004).

The TGF-β pathway has a complex relationship with temperature. *daf-3* mutants are Daf-d when a synchronized population of animals is grown at 25°C, the temperature at which most Daf-c mutants promote dauer arrest (Thomas et al., 1993). However, at 27°C, a temperature at which wild-type animals begin to arrest as dauer larvae at low penetrance, *daf-3* mutants have Daf-c phenotypes (Ailion & Thomas, 2003). This suggests that DAF-3 can function to either promote or suppress dauer entry, in response to perception of environmental temperature.

Regulation of the dauer decision by an insulin-signaling pathway

The insulin signaling pathway was originally defined by the Daf-c genes *daf-2* and *daf-23* (which is allelic to *age-1*), which antagonize the Daf-d gene *daf-16* (Gottlieb & Ruvkun, 1994; Riddle et al., 1981; Thomas et al., 1993). The Daf-d phenotype of *daf-16* null mutants is epistatic to the Daf-c phenotypes of *daf-2* and *age-1*, suggesting that DAF-2 and AGE-1 promote reproductive development by inhibiting the dauer promoting activity of DAF-16 (Gottlieb & Ruvkun, 1994). The Daf-c phenotypes of *daf-2* and *daf-23* are not suppressed by *daf-3*/SMAD mutations, and *daf-16* mutation does not prevent dauer arrest in Daf-c TGF-β pathway mutants, demonstrating that the insulin signaling and TGF-β pathways act in parallel (Gottlieb & Ruvkun, 1994; Vowels & Thomas, 1992). Strains carrying null mutations in *daf-2* cause maternally rescued embryonic lethality, and arrest as dauers and do not recover, suggesting that the insulin signaling pathway regulates the decision to exit the dauer life stage as well as the decision to undergo dauer arrest (Gems et al., 1998). *daf-2* encodes a predicted transmembrane tyrosine kinase, which is the sole *C. elegans* member of the insulin and insulin-like-growth-factor receptor family (Kimura et al., 1997). *age-1* encodes a phosphoinositide-3-kinase (Morris et al., 1996). *daf-16* encodes the sole *C. elegans* member of the FoxO family of transcription factors (Lin et al., 1997; Ogg et al., 1997).

The observation that FoxO family transcription factors interact genetically with insulin signaling in *C. elegans* led to experiments demonstrating that growth factor signaling regulated the nuclear localization of both nematode and mammalian FoxO family members, and rapid characterization of a conserved signaling pathway by which insulin

signaling regulates FoxO activity (Brunet et al., 1999; Brunet et al., 2002; Lee et al., 2001; Lin et al., 2001). Insulin family receptors are obligate disulfide linked homodimers; when bound by ligand and activated, insulin receptor subunits phosphorylate each other and phosphorylate insulin receptor substrate one (IRS-1). Activated IRS-1 allosterically activates phospoinositide-3-kinase, which phosphorylates plasma membrane phosphoinositides, recruiting the kinases PDK-1, AKT-1 and AKT-2 to the plasma membrane. PDK-1 phosphorylates and activates AKT-1 and AKT-2 which phosphorylate FOXO family members at conserved RxRxxS/T sites. Phosphorylated DAF-16/FoxO is sequestered in the cytoplasm through a mechanism which requires the 14-3-3 proteins PAR-5 and FTT-2 and is ubiquitinated and degraded through a mechanism which requires the E3 ubiquitin ligase RLE-1 (Berdichevsky et al., 2006; Li et al., 2007a; Li et al., 2007b). Mutants in the phosphoinositide 3 phosphatase *daf-18*/PTEN which antagonizes AGE-1/PI3K, have Daf-d phenotypes that suppress the Daf-c phenotypes of *age-1* and *daf-2* loss of function mutants (Gil et al., 1999; Mihaylova et al., 1999; Ogg & Ruvkun, 1998). *pdk-1* mutants have *daf-16/FoxO*-dependent dauer constitutive phenotypes at 25°C (Paradis & Ruvkun, 1998). Mutants in *akt-1* have *daf-16/FoxO*-dependent Daf-c phenotypes at 27°C (Hu et al., 2006; Zhang et al., 2008; Ailion & Thomas, 2003). *akt-2* mutation dramatically enhances the penetrance of the Daf-c phenotype of *akt-1* mutants, causing *akt-1;akt-2* mutants to arrest as dauer larvae at 25°C, but does not have a Daf-c phenotype in isolation, suggesting that *akt-1* and *akt-2* act partially redundantly in dauer regulation (Alam et al., 2010; Oh et al., 2005; Paradis et al., 1999; Paradis & Ruvkun, 1998). Strong loss of function mutations in *par-5* and *ftt-2* are lethal; however, RNAi-mediated inactivation of these genes enhances the Daf-c

phenotypes of other DAF-16 activating mutants (Alam et al., 2010; Berdichevsky et al., 2006; Hertweck et al., 2004).

Like TGF-β pathway components, insulin signaling pathway proteins and DAF-16 are broadly expressed. However, rescue experiments using transgenic mosaic animals, or transgenic expression under heterologous promoters, suggest that insulin signaling genes and DAF-16 non-cell autonomously, and likely predominantly in neurons, to regulate dauer arrest (Apfeld & Kenyon, 1998; Wolkow et al., 2000).

Regulation of dauer entry by insulin family ligands

DAF-2 activity is regulated by insulin family ligands. The *C. elegans* genome encodes 40 insulin like (INS) peptides (Li et al., 2003; Pierce et al., 2001). Genetic studies of insulin family genes in *C. elegans* are complicated by likely functional redundancy of insulin family members, and by possible effects of insulin protein overexpression on processing of other insulin family members. Nonetheless, there is evidence that worm insulin-like-peptides regulate dauer arrest and can both activate and inactivate the insulin receptor. A single semi-dominant Daf-c mutation in *daf-28* is the only member of the insulin family that has come out of genetic screens for dauer regulators (Li et al., 2003; Malone & Thomas, 1994) . A *daf-28* promoter drives GFP expression in the amphid neurons ASI and ASJ and is downregulated in response to pheromone and starvation (Li et al., 2003). Similarly, to *daf-28*, RNAi against *ins-7* also enhances the Daf-c phenotype of *daf-2(e1370)* mutants, suggesting that, similar to DAF-28, INS-7 activates DAF-2/IGFr to promote reproductive development (Murphy et al., 2003). Mutations in *ins-4* and *ins-6*

enhance the Daf-c phenotype of *daf-28* mutants, suggesting that *ins-4*, *ins-6*, and *ins-28* act partially redundantly to promotes reproductive development (Cornils et al., 2011; Hung et al., 2014). A loss-of-function mutation in *ins-9* promotes dauer arrest, while INS-9 overexpression blocks some DAF-16-dependent Daf-c phenotypes, suggesting that INS-9 also activates DAF-2 to promote reproductive development (Delaney et al., 2017). In contrast, overexpression of INS-1 and INS-18 enhance dauer arrest in wild-type and *daf-2*/IGFr mutant backgrounds, and *ins-1* and *ins-18* mutations partially suppress the Daf-c phenotypes of *ins-4;ins-6;daf-28* compound mutants, suggesting that these peptides bind to DAF-2/IGFr and inhibit its activity, to promote DAF-16/FoxO activity and dauer entry (Hung et al., 2014; Pierce et al., 2001).

The *eak* pathway regulates dauer arrest and DAF-16/FoxO activity in parallel to *akt-1*

Multiple lines of evidence suggest that the insulin signaling pathway can regulate DAF-16/FoxO activity through mechanisms other than the canonical PI3K-PDK-AKT-DAF-16/FoxO pathway, and independently of changes DAF-16/FoxO nuclear localization. The transcription activating activity of a constitutively nuclear murine Foxo1 lacking its nuclear export signal can be inhibited by addition of insulin to the culture medium to the same extent as the activity of wild-type Foxo1, without causing its nuclear export, suggesting that growth factor signaling inhibits FOXO activity in the nucleus (Tsai et al., 2003). Similarly, in *C. eleg*ans, a transgenically expressed DAF-16 protein in which all three AKT-1 phosphorylation sites are mutated to alanine, is constitutively localized to the nucleus and rescues dauer arrest in *daf-2;daf-16* compound mutants, but does not have a Daf-c phenotype in wild-type animals, suggesting that DAF-2 activity can inhibit

the activity of nuclear DAF-16 independently of AKT phosphorylation and nuclear localization (Berdichevsky et al., 2006; Hertweck et al., 2004; Lin et al., 2001). Activating mutations in *akt-1* and *pdk-1*, and the weak *daf-18*/PTEN allele *daf-18(e1375)* suppress the Daf-c phenotype of *age-1*/PI3K null mutants, but not the Daf-c phenotype of strong loss of function mutants in *daf-2*/IGFr mutants, suggesting that DAF-2 promotes reproductive development through outputs that are independent of AGE-1/PI3K (Gil et al., 1999; Gottlieb & Ruvkun, 1994; Inoue & Thomas, 2000a; Ogg & Ruvkun, 1998; Paradis et al., 1999; Paradis & Ruvkun, 1998; Vowels & Thomas, 1992).

To identify mutations that increase DAF-16/FoxO activity in parallel to AKT-1, a forward genetic screen was performed to identify mutants that cause a Daf-c phenotype in an *akt-1* mutant background at 25°C rather than 27°C (Hu et al., 2006). The genes identified in this screen are named *eak* for enhancers of *akt-1* dauer arrest. The *eak* screen identified twenty-one alleles in seven complementation groups, six of which (*eak-2* through *7*) have been molecularly characterized (Alam et al., 2010; Dumas et al., 2010; Hu et al., 2006; Patel et al., 2008; Williams et al., 2010; Zhang et al., 2008). *eak-2* is allelic to *hsd-1* and encodes a predicted hydroxysteroid dehydrogenase that functions in regulation of the nuclear hormone receptor DAF-12 (see below) activity and regulates expression of DAF-16 target genes in a DAF-12-dependent manner, suggesting that two major transcription factors regulating the dauer decision coordinate to control expression of shared target genes (Dumas et al., 2010). *eak-3* encodes a novel protein (Zhang et al., 2008). *eak-4* encodes a novel protein with an N-myristoylation motif (Hu et al., 2006). *eak-5* is allelic to *sdf-9*, which was identified in another screen for mutations that cause synthetic dauer formation in a sensitized genetic background and encodes a predicted

catalytically inactive phosphatase-like molecule (Hu et al., 2006; Ohkura et al., 2003). *eak-6* encodes another catalytically inactive phosphatase-like molecule (Hu et al., 2006). *eak-7* encodes a conserved protein with an N-myristoylation motif and a conserved TLDC domain, and is expressed in an operon with the heat-shock binding protein HSB-1 (Alam et al., 2010)

The upstream regulatory regions of *eak-2-5* drive expression exclusively in a pair of cells in the head, designated the XXX cells (Dumas et al., 2010; Hu et al., 2006; Zhang et al., 2008). The *eak-6* promoter drives expression in XXX cells and the M1 pharyngeal motor neuron (Hu et al., 2006). A promoter upstream of the *hsb-1/eak-7* operon drives expression in multiple tissues, including neurons, intestine, pharynx, muscle, hypodermis, and a group of cells near the anus (Alam et al., 2010). Heterologous expression of an EAK-7::GFP transgene under a neuronal *ric-19* promoter, or exclusively in the XXX cells under the *eak-4* promoter, rescues the Daf-c phenotype of *eak-7;akt-1* mutants (Alam et al., 2010). These results suggest that all six characterized *eak* genes influence the dauer decision in a non-cell-autonomous manner, with the caveat that this interpretation is based, in part, on overexpression of EAK-7::GFP under heterologous promoters that could drive low-level expression in unforeseen tissues (Alam et al., 2010). Laser ablation of the XXX cells causes a partially penetrant Daf-c phenotype at 25°C, establishing that the XXX cells regulate the dauer decision non-cell-autonomously (Ohkura et al., 2003).

In animals that are wild-type for *akt-1, eak-2-7* mutants develop reproductively at 25°C but have Daf-c phenotypes at 27°C, suggesting that *eak* mutations can promote dauer

formation when the *akt-1* branch of the insulin signaling pathway is active (Alam et al., 2010; Dumas et al., 2013b; Hu et al., 2006; Zhang et al., 2008). The Daf-c phenotypes of compound mutants carrying any combination of two *eak* mutations are not more penetrant than the Daf-c phenotypes of single mutants, suggesting that the *eak* genes act in a single genetic pathway to regulate dauer arrest. The Daf-c phenotypes of all *eak* mutations are blocked by the addition of a *daf-16* null mutation, and *eak* mutants display *daf-16*-dependent transcriptional upregulation of canonical *daf-16* target genes, suggesting that the EAK pathway acts to inhibit DAF-16/FoxO activity. *eak* mutants are suppressed by *daf-12*/NHR mutants, confirming that EAK pathway acts upstream of the nuclear hormone pathway (Alam et al., 2010; Dumas et al., 2010; Hu, 2007; Zhang et al., 2008).

EAK-3-7 are localized to the plasma membrane and control DAF-16/FoxO activity in the nucleus, suggesting that they influence DAF-16/FoxO indirectly. EAK-7 encodes a conserved protein that has a similar expression pattern to DAF-16, and controls DAF-16 transcriptional activity in multiple tissues (Alam et al., 2010). A predicted null allele of *eak-7* increases levels of DAF-16 protein by Western blot and causes DAF-16-dependent increases in lifespan in addition to its dauer arrest phenotypes (Alam et al., 2010). These characteristics suggest that EAK-7 may be a downstream component of the *eak* pathway and indicate that it may be useful for identifying gene products that transduce signals to DAF-16 in the nucleus. To identify possible downstream genes affecting nuclear DAF-16/FoxO activity, a forward genetic screen was conducted for mutations which suppress the 25°C dauer-constitutive phenotype of *eak-7;akt-1* compound mutants, or *seak* genes (Dumas et al., 2013a). In principle *seak* genes may act in the *eak* pathway to control

activity of nuclear DAF-16/FoxO, in the canonical insulin signaling pathway to affect DAF-16/FoxO localization, or downstream of or in parallel to DAF-16. The *seak* screen has identified mutations in two genes, *dpy-21* and *set-4*, that have been previously reported to act in the dosage compensation pathway (Wells et al., 2012; Yonker & Meyer, 2003). Both *dpy-21* and *set-4* mutations appear to suppress *eak-7;akt-1* dauer arrest by causing differential regulation of X-linked insulin signaling pathway genes, including *ins-9* and *akt-2* (Delaney et al., 2017; Dumas et al., 2013a). The *seak* screen has also identified a large rearrangement on the X-chromosome, which may suppress the *eak-7;akt-1* Daf-c phenotype by duplicating *akt-2*, and a novel allele of *daf-12* (Itani et al., 2016a; Itani et al., 2016b).

Dauer regulatory signals converge on the nuclear hormone receptor DAF-12

A steroid hormone signaling pathway that regulates dauer arrest was originally defined by Daf-c mutations in the gene *daf-9* and Daf-d mutations in the gene *daf-12*. *daf-12* null mutants do not form dauer larvae in starved, high population density cultures, and are epistatic to all known Daf-c mutants, suggesting that sensory perception, TGF-β signaling and insulin signaling converge on DAF-12 activity to regulate dauer entry (Albert & Riddle, 1988; Antebi et al., 2000; Fielenbach & Antebi, 2008; Gerisch et al., 2001; Riddle et al., 1981; Thomas et al., 1993). *daf-12* encodes a nuclear hormone receptor, which includes a ligand binding and DNA binding domain (Antebi et al., 2000). While *daf-12* null mutants are Daf-d, mutations within the *daf-12* ligand binding domain have Daf-c phenotypes, suggesting that unliganded DAF-12 promotes dauer arrest (Antebi et

al., 2000). DAF-12::GFP is expressed in the nucleus in most cells, suggesting that it may act cell-autonomously to promote dauer-specific developmental programs (Antebi et al., 2000).

Bile acid-like steroids, including Δ^7- and $\Delta^{1,7}$-dafachronic acid, have been chemically identified as DAF-12 ligands (Mahanti et al., 2014; Motola et al., 2006). DAF-9 encodes a cytochrome P450 family member that is required for biosynthesis of both dafachronic acid species (Gerisch et al., 2001; Jia et al., 2002; Mahanti et al., 2014; Motola et al., 2006) . The Daf-c phenotypes of *daf-9* mutants are rescued by administration of exogenous dafachronic acids, indicating that DAF-9 antagonizes the dauer promoting activity of DAF-12 by promoting dafachronic acid biosynthesis (Mahanti et al., 2014; Motola et al., 2006).

In addition to *daf-9,* three other genes, *hsd-1*, *daf-36,* and *dhs-16* have been proposed to act in the dafachronic acid biosynthesis pathway. *daf-36* encodes an enzyme with homology to Rieske oxygenases, *hsd-1* encodes a hydroxysteroid dehydrogenase family member, and *dhs-16* encodes an enzyme with homology to short chain dehydrogenases (Dumas et al., 2010; Patel et al. 2008; Rottiers et al. 2006; Wollam et al. 2012). *daf-36, hsd-1,* and *dhs-16* mutants have less penetrant Daf-c phenotypes than *daf-9* mutants, with *daf-36, hsd-1,* and *dhs-16* mutants undergoing dauer arrest in synchronized cultures at 27°C but not at lower temperatures, whereas *daf-9* null mutants are dauer-constitutive regardless of temperature (Dumas et al., 2010; Gerisch et al., 2001; Jia et al., 2002; Patel et al., 2008; Rottiers et al., 2006; Wollam et al., 2012). Compound *hsd-1;daf-36, dhs-16;daf-36,* and *dhs-16;hsd-1* mutants have an enhanced dauer arrest phenotype at 25°C

suggesting that these enzymes may act partially redundantly or in parallel (Dumas et al., 2010; Patel et al., 2008; Wollam et al., 2012). Biochemical analysis has revealed small decreases in levels of Δ^7- and $\Delta^{1,7}$- dafachronic acids in *hsd-1;daf-22* mutants, relative to *daf-22* controls (which have high levels of dafachronic acids likely due to their defects in dauer pheromone biosynthesis), along with the appearance of new steroid molecules which may be dafachronic acid precursors (Mahanti et al., 2014). In contrast, Δ^7- and $\Delta^{1,7}$- dafachronic acids appear to be absent in *daf-36;daf-22* mutants (Mahanti et al., 2014). *daf-36;daf-22* mutants do produce a dafachronic acid precursor (Δ^0-DA) the presence of which is correlated with activity in suppressing *daf-9* dauer arrest, potentially accounting for the less penetrant Daf-c phenotype of *daf-36* mutants relative to *daf-9* (Mahanti et al., 2014). *dhs-16* mutation greatly reduced levels of Δ^7-dafachronic acid and causes a more modest reduction in $\Delta^{1,7}$-dafachronic acid levels (Mahanti et al., 2014). These data are consistent with a model whereby DAF-36 and DHS-16 act in the same pathway as DAF-9 to produce dafachronic acids, while HSD-1 either modulates this pathway, potentially in a cell type specific matter, or regulates dauer arrest through an independent mechanism.

Availability of exogenous dafachronic acids provided the opportunity to test whether dafachronic acid biosynthesis functions downstream of the TGF-β and insulin signaling in regulation of dauer entry. Treatment of *daf-9*/cytochromeP450 mutants with Δ^7-dafachronic acid during a specific temporal window (15 to 33 hours post hatch) within the L2d larval stage rescued dauer arrest in a dose dependent manner, suggesting that a critical threshold of dafachronic acid must be reached during the L2d stage to commit to reproductive development (Schaedel et al., 2012). Consistent with the observations that

forward genetic screens for suppression of TGF-β pathway mutants yield *daf-12* mutations, treatment with Δ^4-dafachronic acid rescued the dauer formation constitutive phenotype of *daf-7*/TGF-β mutants, confirming that dafachronic acids act downstream of or in parallel to the TGF-β pathway, and suggesting that DAF-7/TGF-β signaling may regulate DAF-12 activity by regulating enzymes that synthesize dafachronic acids (Motola et al., 2006). Treatment of strong *daf-2* loss of function mutants with Δ^4-dafachronic acid also prevents formation of fully remodeled dauer larvae; however, animals do arrest development as L3 larva (Motola et al., 2006). Interestingly, this L3 developmental arrest is also observed when some strong loss of function alleles of *daf-2*/IGFr are crossed into *daf-12(0)* backgrounds (Vowels & Thomas, 1992). These observations are consistent with a model in which reduced insulin signaling promotes dauer arrest in part by reducing levels of DAF-12 ligands, and in part through DAF-12-independent activities of DAF-16/FoxO. Studies showing that DAF-16 is required for dauer larva to suppress cell differentiation and cell division in the vulva, and for normal vulval morphogenesis when dauer larva resume reproductive development are also consistent with the hypothesis that DAF-16/FoxO has DAF-12-independent roles in dauer development (Karp & Greenwald, 2013). The role of DAF-16 in other forms of non-dauer diapause, including the developmental arrest of starved L1 larvae, together with observations of DAF-12/NHR independent L3 arrest in strong *daf-2*/IGFr mutants, suggests that DAF-16/FoxO may promote cessation of developmental cell division programs during diapause states in a DAF-12-independent manner, while DAF-12 is required for dauer-specific developmental patterning events (Baugh & Sternberg, 2006; Motola et al., 2006; Vowels & Thomas, 1992). The observation that the *hsd-1* Daf-c

phenotype is *daf-16*-dependent and that *hsd-1* mutants exhibit upregulation of direct transcriptional targets of DAF-16 in a DAF-12 dependent manner is also more consistent with models in which DAF-16 and DAF-12 coordinate and regulate each other's activity, than with models in which DAF-12 is simply downstream of DAF-16 (Dumas et al., 2010; Patel et al., 2008).

Unlike DAF-12, which is broadly expressed, expression of the Daf-c genes encoding dafachronic acid biosynthesis enzymes is cell-type specific. A rescuing DAF-9::GFP construct driven by the endogenous *daf-9* promoter is expressed in the XXX cells, in the hypodermis of L2 to L4 larva undergoing reproductive development, and in the spermatheca of adult hermaphrodites (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). A rescuing HSD-1::GFP construct driven by the endogenous promoter is expressed exclusively in the XXX cells (Patel et al., 2008). A rescuing DAF-36::GFP construct driven by its endogenous promoter is expressed in the intestine, a pair of unidentified (non-XXX) neurons in the head, the head mesodermal cell, and the hypodermal seam cells of dauer larvae (Rottiers et al., 2006). It has been suggested that the tissue distribution of dafachronic acid biosynthesis genes allows inputs from multiple tissues to influence dafachronic acid production and dauer entry (Fielenbach & Antebi, 2008). DAF-9::GFP expression is absent in the hypodermis of dauer larva of *daf-2*/IGFr and *daf-7*/TGF-β mutants, though expression persists in dauer XXX cells (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). DAF-9::GFP expression is absent in the hypodermis of dauer larvae of *daf-2*/IGFr and *daf-7*/TGF-β mutants, though expression persists in dauer XXX cells (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). Constitutive expression of DAF-9 under the hypodermal *dpy-7* promoter fully suppressed dauer arrest in *daf-7*

mutants and caused non-dauer L3 arrest in strong *daf-2* loss-of-function mutants, suggesting that Daf-c mutants in both TGF-β and insulin signaling pathways promote dauer arrest, at least in part, by downregulating DAF-9 expression (Mak & Ruvkun, 2004). Interestingly, DAF-9::GFP expression increased, in a DAF-12-dependent manner, during reproductive development under mildly dauer-inducing conditions, such as culture of *daf-7*/TGF-β and *daf-2*/IGFr mutants at 20°C, or treatment with sub-dauer inducing concentrations of pheromone (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). This observation may be consistent with a model where accumulation of DAF-12 ligands promotes progression from L2d to the reproductive life history under conditions that are too stressful for commitment to reproductive development at L1, but not sufficiently stressful to induce commitment to the dauer life history. DAF-36::GFP expression is downregulated in *daf-2* mutants but not in TGF-β or guanylyl cyclase pathway Daf-c mutants, suggesting that reduced insulin signaling may promote dauer arrest, in part, by downregulating DAF-36 levels (Rottiers et al., 2006). Effects of Daf-c mutations on HSD-1::GFP expression have not yet been reported. Taken together, the data is consistent with a model where guanylyl cyclase, TGF-β and insulin signaling control dauer entry by regulating biosynthesis of dafachronic acids, though the precise molecular events that lead from reduced insulin and TGF-β signaling to lower levels of dafachronic acid biosynthesis enzymes remain to be determined.

DAF-12/NHR is regulated by direct interaction with DIN-1/SHARP

Nuclear hormone receptors often mediate transcriptional activation and repression by assembling complexes with co-activator and co-repressor proteins that potentiate

transcription by promoting histone acetylation, or block transcription by promoting histone de-acetylation (Fielenbach & Antebi, 2008). Unliganded DAF-12 assembles with DIN-1, a homologue of the human SHARP co-repressor, to promote dauer formation (Ludewig et al., 2004). Biochemical interaction between DAF-12 and DIN-1 was first identified by yeast two-hybrid (Ludewig et al., 2004). DIN-1 is expressed in two transcriptional isoforms, DIN-1S and DIN-1L. Mutations that specifically affect the DIN-1S isoform emerged from forward genetic screens for suppression of gonadal migration phenotypes of DAF-12 ligand binding domain mutants. Mutants that affect the DIN-1L isoform have a distinct set of unrelated developmental phenotypes, which may explain why DIN-1 alleles have not previously come out of dauer suppression screens (Ludewig et al., 2004). *din-1s* mutants suppress the Daf-c phenotypes of *daf-9* and *daf-12* ligand binding domain mutants, indicating that DIN-1S is required for dauer formation downstream of or parallel to unliganded DAF-12. *din-1s* mutants also suppress the Daf-c phenotypes of *daf-7*/TGF-β mutants and of weaker *daf-2*/IGFr alleles (*e1368*, *e1369*, and *m41*) but do not suppress the Daf-c phenotypes of stronger *daf-2* alleles (*e1370* and *e1391*), consistent with a model in which TGF-β pathway mutants are fully dependent on DIN-1 to promote dauer arrest, while reduced insulin signaling can promote dauer arrest through DIN-1-dependent and -independent mechanisms (Ludewig et al., 2004).

Regulation of dauer arrest by heterochronic genes

While unliganded DAF-12 is required for dauer entry, liganded DAF-12 plays a role in promoting developmental programs specific to the L2 and L3 larval stages of reproductive development. Mutations that disrupt dafachronic acid biosynthesis or

binding to DAF-12 have defects in gonadal development, establishing a well characterized role for liganded *daf-12* in gonad morphogenesis during non-dauer reproductive development (Antebi et al., 1998; Antebi et al., 2000; Jia et al., 2002). Mutations in the DAF-12 DNA binding domain cause repetition of L2 stage specific developmental programs in multiple tissues after the transition to the L3 larval stage, demonstrating that DAF-12 plays a role in L2 to L3 transitions during normal reproductive development, as well as during dauer formation (Antebi et al., 1998; Antebi et al., 2000).

In *C. elegans*, a network of genes called heterochronic loci, which includes *daf-12*, acts in multiple tissues to control developmental events that are specific to each larval stage (Moss, 2007). Mutations in heterochronic genes alter the sequence of developmental stage-specific programs, either skipping stage-specific programs in favor of early expression of later developmental stage or adult programs (termed a precocious phenotype) or repeating expression of stage-specific programs (termed a retarded, or delayed phenotype) (Fielenbach & Antebi, 2008; Moss, 2007). For example, mutations in the heterochronic gene *lin-4*, which encodes the first discovered microRNA, cause repetition of L1 developmental programs, including division of intestinal nuclei which normally occurs only at L1, and L1-specific patterns of hypodermal cell divisions at each subsequent larval stage (Chalfie et al., 1981; Moss, 2007; Lee & Ambros, 2003). Somatic developmental programs that are specific to larval stages after L1 rarely occur in *lin-4* mutants, though germline maturation occurs at the normal time in *lin-4* and other heterochronic mutants (Chalfie et al., 1981; Moss, 2007). Genetic epistasis analysis of heterochronic phenotypes has put the heterochronic genes into a regulatory hierarchy;

some but not all of these genetic interactions are understood molecularly (Fielenbach & Antebi, 2008; Moss, 2007). Recent work has established that multiple components of the heterochronic pathway affect the dauer decision through mechanisms that involve genetic or molecular interactions with DAF-12 (Karp & Ambros, 2011; Tennessen et al., 2010).

LIN-42 is homologous to the circadian transcription factor *period*. It is expressed cyclically during larval development with peak expression within larval stages, while expression is reduced at molts, when animals are transitioning between larval stages (Tennessen et al., 2006). Expression of *lin-42* is downregulated after molting during the dauer diapause (Tennessen et al., 2006; Tennessen et al., 2010). Like DAF-12, LIN-42 is important for hypodermal patterning during the L3 larval stage and proper development of the somatic gonad (Tennessen et al., 2006; Tennessen et al., 2010). *lin-42* mutants have a Daf-c phenotype when cultured at 27°C, and LIN-42 overexpression during the L2 larval stage suppresses the Daf-c phenotypes of insulin signaling, TGF-β, dafachronic acid biosynthesis, and *daf-12* ligand binding domain mutants (Tennessen et al., 2010). LIN-42 protein directly binds DAF-12 in yeast, suggesting that that LIN-42 may regulate DAF-12 transcriptional activity via a direct physical interaction (Tennessen et al., 2010).

The *hunchback* like transcription factor HBL-1 acts in opposition to DAF-12 to promote L2-specific developmental programs and inhibit L3-specific programs; *hbl-1* mutants have precocious phenotypes in which L2 developmental programs are skipped and L3-specific developmental programs are expressed in L2 larvae, while in *daf-12* DNA binding domain mutants L2 specific developmental programs are repeated at the L3 larval stage (Abrahante et al., 2003; Bethke et al., 2009). Two independent loss-of-

function alleles of *hbl-1* suppress the Daf-c phenotype of a *daf-12* ligand binding mutant (Karp & Ambros, 2011). This suggests that *hbl-1* promotes dauer entry, either through ligand-independent regulation of DAF-12, or through regulation of shared DAF-12/HBL-1 target genes. Interestingly the same alleles enhance dauer-constitutive phenotypes of insulin and TGF-β pathway mutants, suggesting that HBL-1 regulates dauer arrest through distinct, possibly developmental stage-specific activities (Karp & Ambros, 2011).

The transcription factor LIN-14 has been placed genetically upstream of DAF-12 in the heterochronic pathway (Antebi et al., 1998). LIN-14 is expressed in fasted L1 larva and promotes expression of L1 and L2 specific programs in many somatic tissues (Ambros & Horvitz, 1987; Reinhart & Ruvkun, 2001; Ruvkun et al., 1989; Ruvkun & Giusto, 1989). Upon feeding *lin-14* expression is downregulated by interaction with the micro-RNA *lin-4*, resulting in decreasing levels of LIN-14 protein throughout the L1 larval stage (Bagga et al., 2005; Ha et al., 1996; Lee et al., 1993; Olsen & Ambros, 1999; Wightman et al., 1993). Loss-of-function mutants in *lin-14* skip L1-specific developmental programs and express L2-specific programs precociously, while constitutive expression of LIN-14 caused by *lin-4* mutation or mutation of the *lin-4* binding site in the LIN-14 3'UTR results in repeated expression of L1-specific larval programs (Ambros & Horvitz, 1987; Olsen & Ambros, 1999). Interestingly, when placed into Daf-c mutant backgrounds, *lin-14* mutants also arrest as partially remodeled dauer larvae at the L2 larval stage rather than at L3 (Liu & Ambros, 1989). In contrast, *lin-4* mutants are incapable of forming dauer larvae in starved cultures(Liu & Ambros, 1989). These results are consistent with a model wherein LIN-14 inhibits DAF-12 activity during dauer development as well as

during reproductive development and permits expression of dauer developmental programs at the L2 to L3, but not the L1 to L2 transition. Alleles of *lin-14* that impact the direction, rather than the timing, of the dauer decision have not been previously identified.

Conclusions and Future Directions

The *C. elegans* dauer decision was initially intended to serve as an example of developmental plasticity, which could serve to elucidate potentially conserved signaling pathways governing developmental decisions. Over the last 40 years studies of the dauer decision have revealed a surprisingly intricate signal transduction network regulating dauer arrest. Perception of environmental factors is integrated in the nervous system, to regulate the activity of two major, highly conserved signal transduction cascades (the insulin signaling and TGF-β pathways), that converge on a nuclear hormone receptor DAF-12. DAF-12 acts as a ligand dependent environmental switch that can promote developmental programs specific to either the dauer diapause or reproductive life histories.

Despite the high levels of molecular detail that have emerged from existing studies of the genetics of dauer formation, many key questions remain to be answered. Mechanisms by which DAF-12 and DAF-16/FoxO coordinate to specify the dauer life history remain to be established, along with the overlapping and unique outputs of these transcription factors that enable the fascinating ability of dauer larvae to halt all developmental

programs for many times the lifetime of an adult animal, and then resume normal development as though nothing had occurred. The ability of passage through the dauer larval stage to rescue aberrant precocious differentiation in some heterochronic mutants suggests that a fuller understanding of the quiescence that occurs in dauer larva may illuminate conserved mechanisms governing quiescence and cell differentiation, as well as novel molecular mechanisms underlying post-dauer development (Karp & Ambros, 2012).

Signal transduction pathways that regulate the dauer decision are not fully understood. Continued forward genetic screens in sensitized environments or genetic backgrounds may continue to reveal novel modulators of dauer arrest with conserved functions in insulin, TGF-β or steroid hormone signaling, as well as interactions between the dauer decision and other developmental mechanisms. These studies will be aided by advancements in whole genome sequencing and the availability of CRISPR Cas9-based gene editing to perform detailed genetic analysis of identified genes (Hu, 2014; Paix et al., 2015). More broadly, physiological studies are needed to fully understand the neural circuits which integrate environmental inputs to produce the dauer decision, and to understand when each step in the dauer decision signal transduction cascade acts to specify commitment to the L2d larval stage and then to undergo the dauer life history. It seems highly likely that studies of the "simple" genetic switch governing *C. elegans* dauer formation will continue to provide insights into conserved mechanisms of development and plasticity for years to come.

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Chapter 2 A novel mutation in *lin-14* suppresses dauer arrest

Introduction

In the nematode *C. elegans*, the insulin like growth factor receptor homologue DAF-2 promotes reproductive development by inhibiting activity of the FoxO transcription factor DAF-16 (Fielenbach & Antebi, 2008). Under replete conditions, nematodes develop directly to adulthood by traversing four larval stages (L1-L4) after hatching (Brenner, 1974; Stiernagle, 2006). Under conditions of increased temperature, high population density, and low food availability L1 larvae can commit to an alternate life history, during which they pass through a larval diapause called dauer. Loss-of-function mutations in components of the canonical insulin signaling pathway, including the insulin-like-growth-factor-receptor family-member *daf-2*, the predicted phosphoinositide-3-kinase *age-1*, *pdk-1* and *akt-1*, all have dauer constitutive phenotypes when cultured at 25°C or 27°C(Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997; Paradis et al., 1999; Paradis & Ruvkun, 1998). The dauer-constitutive phenotypes of *daf-2*/IGFr mutants are fully suppressed by null mutations of the sole *C. elegans* member of the FoxO transcription factor family, *daf-16*, suggesting that activity of the *C. elegans* insulin signaling pathway promotes reproductive development by inhibiting DAF-16 activity (Gottlieb & Ruvkun, 1994; Vowels & Thomas, 1992).

Activity of the insulin signaling pathway inhibits DAF-16/FoxO activity, in part, by triggering phosphorylation of DAF-16/FoxO by the serine threonine kinases AKT-1 and AKT-2, at conserved RxRxxS/T sites, which leads to DAF-16 being exported from the nucleus and degraded (Berdichevsky et al., 2006; Brunet et al., 1999; Brunet et al., 2002; Hertweck et al., 2004; Lin et al., 2001). However, translocation to the nucleus is not sufficient to fully activate DAF-16/FoxO, or mouse FOXO1, suggesting that DAF-2/IGFr also regulates nuclear DAF-16/FoxO in parallel to canonical insulin signaling (Lin et al., 2001; Tsai et al., 2003). Forward genetic screens for mutations that enhance the dauer arrest phenotype of *akt-1* mutants (or *eak* mutations) identified components of the *eak* pathway, which regulate activity of nuclear DAF-16/FOXO (Alam et al., 2010; Dumas et al., 2010; Hu et al., 2006; Williams et al., 2010; Zhang et al., 2008). EAK-7 is a conserved plasma membrane protein which regulates the nuclear pool of DAF-16/FoxO protein through unknown intermediary molecules (Alam et al., 2010).

We attempted to identify downstream components of the *eak* pathway by conducting a forward genetic screen for mutations which suppress the 25°C dauer constitutive phenotype of *eak-7;akt-1* compound mutants (Dumas et al., 2013). In principle, *seak* genes could include novel components of the *eak* pathway, novel regulators of canonical insulin signaling, or genes that act downstream of or in parallel to both pathways to regulate dauer arrest. In our previously published work we identified two mutations in dosage compensation pathway genes that suppress *eak-7;akt-1* dauer arrest by causing differential regulation of two X-linked insulin signaling pathway genes, *ins-9* and *akt-2*. We also identified a large rearrangement of the X chromosome which likely suppresses *eak-7;akt-1* dauer arrest by duplicating the insulin signaling pathway gene AKT-2, and a

novel mutation in DAF-12 (Delaney et al., 2017; Dumas et al., 2013; Itani et al., 2016a; Itani et al., 2016b).

Here we describe a novel intronic mutation in the heterochronic gene *lin-14* that was identified in our screen for suppressors of *eak-7;akt-1* dauer arrest. LIN-14 has been previously identified as a component of the heterochronic pathway; it promotes execution of developmental programs specific to the L1 and L2 larval stages (Ambros & Horvitz, 1984, 1987; Reinhart & Ruvkun, 2001; Ruvkun et al., 1989). The *lin-14* transcript is the target of the first discovered interaction between a gene product and a micro-RNA; the micro-RNA *lin-4* binds to the 3'UTR of the LIN-14 transcript and both inhibits its translation and targets it for degradation, resulting in declining levels of the LIN-14 protein throughout the L1 larval stage (Bagga et al., 2005; Ha et al., 1996; Lee et al., 1993; Olsen & Ambros, 1999; Ruvkun et al., 1989; Wightman et al., 1993). While *lin-14* lacks obvious sequence conservation to vertebrate genes, the LIN-14 protein has been validated biochemically as a transcription factor which binds to a consensus GAACRY motif and regulates stage specific expression of target genes downstream of its consensus binding site in vivo (Hristova et al., 2005). LIN-14 acts upstream of DAF-12 in patterning the hypodermis during reproductive development (Antebi et al., 1998). Strong loss of function mutants in *lin-14* enter dauer precociously at the L2 larval stage (Liu & Ambros, 1989). In contrast, *lin-4* mutants, which reiterate L1 specific developmental programs at subsequent larval stages due to constitutive expression of LIN-14, cannot form dauer larvae (Liu & Ambros, 1989). These data suggest that LIN-14 acts during the dauer life history to prevent precocious execution of dauer developmental programs prior to the L3 larval stage (Liu & Ambros, 1989).

Results

A genetic screen for suppressors of *eak-7;akt-1* dauer arrest

The *seak* screen was previously described (Dumas et al., 2013). Compound mutants harboring the predicted null alleles *eak-7(tm3188)* and *akt-1(ok525)* constitutively undergo dauer arrest in developmentally synchronized cultures at 25°C. We reasoned that a forward genetic screen for mutations which could suppress the Daf-c phenotype of *eak-7;akt-1* mutants might identify novel activators of DAF-16/FoxO. Therefore, we mutagenized *eak-7;akt-1* compound mutants with N-ethyl-N-nitrosourea (ENU) and screened for rare F_2 suppressors of the *eak-7;akt-1* Daf-c phenotype (*seak* mutants). We isolated 16 independent *seak* mutant strains and subjected them to whole genome sequencing, and their genomes were compared to an unmutagenized *eak-7;akt-1* strain to identify mutations introduced by ENU mutagenesis. The *eak-7;akt-1;seak* strain harboring the *lin-14(dp69)* mutation was outcrossed to BQ29, a recombinant inbred strain containing *eak-7(tm3188)* and *akt-1(ok525)* mutations that had been specifically introgressed into the polymorphic background CB4856. Single nucleotide polymorphism mapping was used to map the *seak* phenotype to a region on the X chromosome containing *lin-14(dp69)* and two other mutations (*dp68* and *dp70*). These mutations were separated by outcrossing the *eak-7(tm3188);akt-1(ok525);seak* strain to an unmutagenized *eak-7;akt-1* strain on the N2 wild-type background, and dauer assays were used to confirm that *lin-14(dp69)* suppressed *eak-7;akt-1* dauer arrest while the

dp68 and *dp70* mutations did not. This result suggests that *lin-14(dp69)* is the causative suppressor of *eak-7;akt-1* dauer arrest, although we cannot rule out the possibility of a closely linked background mutation that was not identified by whole genome sequencing.

A novel mutation in *lin-14* suppresses *eak-7;akt-1* dauer arrest

lin-14(dp69) is an intronic point mutation in the sixth base pair downstream of the 5' junction of the splice site joining exons five and six in the *lin-14* locus, the first *lin-14* exons that are common to all *lin-14* isoforms (Figure 2.1). *lin-14(dp69)* mutation caused penetrant suppression of the *eak-7;akt-1* Daf-c phenotype (Figure 2.2 panel A, Table 2.2). *lin-14* RNAi also strongly suppressed the *eak-7;akt-1* dauer constitutive phenotype, suggesting that reducing levels of LIN-14 transcript or protein prevents dauer arrest in *eak-7;akt-1* mutants (Figure 2.2 panel B, Table 2.2).

During the course of performing RNAi dauer assays we observed that a much higher percentage of *eak-7;akt-1;lin-14(dp69)* animals underwent dauer arrest when cultured on the *E. coli* strain HT115 (the strain RNAi clones are propagated in) than when cultured on the *E. coli* strain OP50. A direct comparison of *eak-7;akt-1;lin-14(dp69)* animals on RNAi plates seeded with OP50 and HT115 bacteria expressing empty vector RNAi at equal optical densities was consistent with the interpretation that increased dauer arrest in these experiments was caused by culture on the HT115 bacterial strain (Table 2.2).

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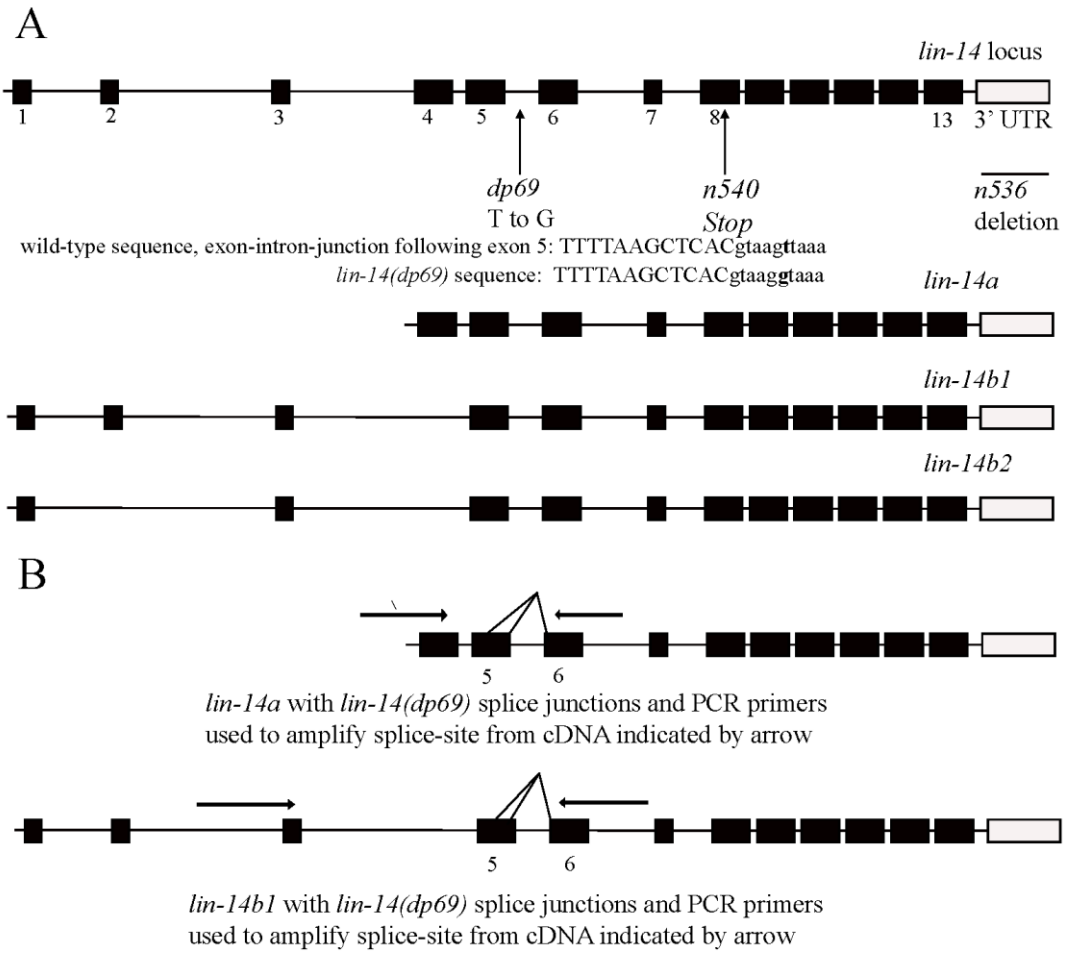
Figure 2.1. *lin-14(dp69)* is a novel *lin-14* allele

A. Structure of the *lin-14* genomic locus, *lin-14* alleles used, sequence of the splice junction between exons five and six in wild-type and *lin-14(dp69)* animals with the mutated base in bold, and *lin-14* transcriptional isoforms. B. Changes in splicing caused by *lin-14(dp69)*: arrows indicate isoform specific primers used to amplify across splice junctions from cDNA, lines indicate alternative splicing sites from the coding sequence of exon five to the exon six splice-acceptor site, resulting in either an out-of-frame or in-frame deletion (see table 2.2 for sequences).



lin-14(dp69) and *lin-14* RNAi additively suppress the dauer constitutive phenotype of *daf-2(e1368)* mutants

Mutations that suppress *eak-7;akt-1* dauer arrest may act in the *eak* pathway, they may be more general regulators of insulin signaling, or they may act downstream of, or in parallel to insulin signaling. To determine whether *lin-14* acts in the *eak* pathway or is a more general regulator of the dauer decision, we measured the effects of *lin-14(dp69)* and *lin-14* RNAi on dauer arrest in other Daf-c mutant backgrounds.

Neither *lin-14(dp69)* nor treatment with *lin-14* RNAi significantly suppressed the dauer constitutive phenotypes of *daf-2(e1368)* mutants. Surprisingly treatment of *daf-2(e1368);lin-14(dp69)* compound mutant animals with *lin-14* RNAi caused an additive and highly penetrant dauer suppression phenotype (Figure 2.2C, Table 2.2). These data suggest that *lin-14(dp69)* and *lin-14* RNAi can suppress the dauer-constitutive phenotypes of insulin signaling mutants and are consistent with a model where LIN-14 promotes dauer arrest independently of the *eak* pathway. The data are also consistent with a model where modulation of *lin-14* transcript levels dramatically impacts the dauer decision, with additive changes in levels of functional *lin-14* transcript triggered by combining *lin-14(dp69)* mutations and *lin-14* RNAi causing a more highly penetrant dauer suppressive phenotype than either intervention alone.

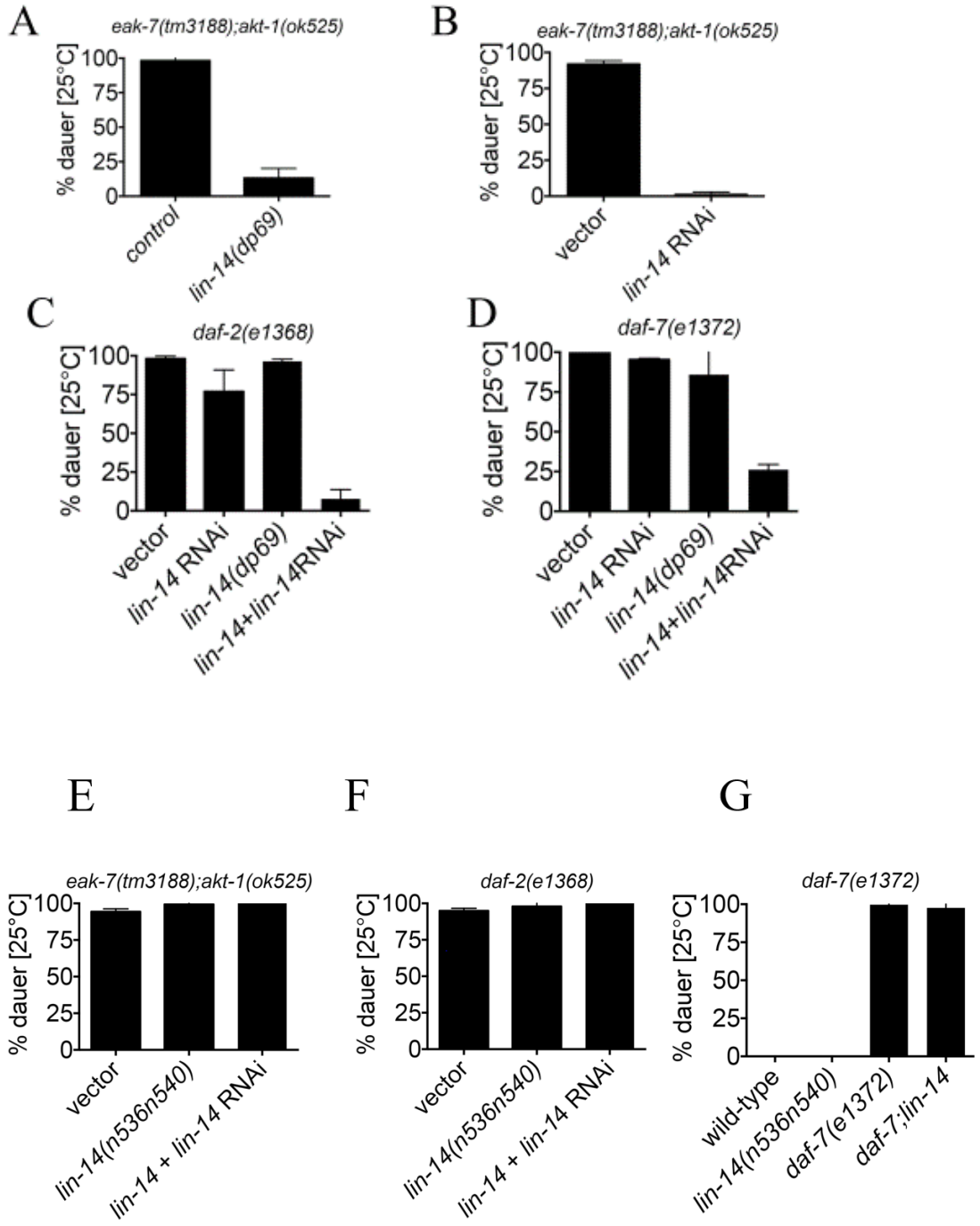
Additive suppression of *daf-7*/TGF-β mutant dauer constitutive phenotypes by *lin-14(dp69)* and *lin-14* RNAi

We next asked whether dauer regulation by *lin-14* was specific to the insulin signaling pathway. We crossed *lin-14(dp69)* into a *daf-2(e1372)* mutant. *daf-7* encodes a

transforming growth factor beta-like peptide, and *daf-7* mutants have a dauer-constitutive phenotype that depends on *daf-3*/SMAD, *daf-5*/SnoN and *daf-12*/NHR and is independent of *daf-16*/FoxO (Patterson & Padgett, 2000; Shi & Massagué, 2003). Neither *lin-14(dp69)* nor *lin-14 RNAi* significantly suppressed the Daf-c phenotype of *daf-7(e1372)* mutants (Figure 2.2 panel D, Table 2.2). We observed highly penetrant suppression of the *daf-7*/TGF-β dauer constitutive phenotype in an experiment in which *daf-7(e1372);lin-14(dp69)* mutants were treated with *lin-14* RNAi (Figure 2.2 panel D, Table 2.1). These data are most consistent with a model in which *lin-14* regulates the dauer decision downstream of or in parallel to both the insulin signaling and TGF-β signaling pathways.

Figure 2.2. Effects of *lin-14* mutations and *lin-14* RNAi on dauer arrest

A-D. Effects of *lin-14(dp69)* and *lin-14* RNAi on dauer entry at 25°C in dauer constitutive backgrounds. E-G. *lin-14(n536n540)* does not suppress dauer arrest. Dauer constitutive genotype is indicated at the top of the panel, X-axis is labeled with additional mutations or RNAi treatments added. See table 2.2 for data.



lin-14(dp69) affects splicing of the *lin-14* transcript

The molecular lesion in *lin-14(dp69)* converts a guanine to thymidine six nucleotides into the intron between exons five and six of *lin-14*, suggesting that it might affect splicing. Exons five and six are the first exons that are shared between all *lin-14* transcriptional isoforms. Isoform-specific mutations in *lin-14* have distinct effects. The LIN-14A isoform is required early in the L1 larval stage to specify L1-specific developmental programs instead of precocious L2 developmental programs. The two LIN-14B isoforms act later in the L1 larval stage to specify L2 specific developmental programs after the next molt, rather than precocious L3 specific programs (Ambros & Horvitz, 1987; Reinhart & Ruvkun, 2001). (Note, the letter designations assigned to LIN-14 isoforms in the current annotation of the publicly available worm genome are reversed from those in the literature: here we use the naming convention established in prior publications (Reinhart & Ruvkun, 2001)). To test the hypothesis that *lin-14(dp69)* affects splicing, and determine whether it has isoform specific-effects, we collected RNA from mixed populations of wild-type and *lin-14(dp69)* animals and performed reverse-transcriptase-PCR across the exon-exon junction predicted to be affected in *lin-14(dp69)* using primer pairs specific to both *lin-14a* and *lin-14b* transcripts. We then cloned and sequenced individual transcripts. All sequenced transcripts from wild-type animals had the expected exon-exon junction (Figure 2.1B, Table 2.1). In fourteen sequenced *lin-14b* transcripts and nine *lin-14a* transcripts from *lin-14(dp69)* cultures, a novel splice donor site in exon five was spliced to the canonical splice acceptor site in exon six, resulting in a five amino acid deletion followed by a frameshift and multiple early stop codons. In an additional *lin-14a* transcript and two *lin-14b* transcripts, splicing from a different donor site in exon

five to the canonical splice acceptor site in exon six resulted in a predicted in-frame deletion of nine amino acids followed by a proline to alanine conversion. This is consistent with a model where the *lin-14(dp69)* mutation reduces LIN-14 transcript and protein levels by nonsense mediated decay of affected transcripts, while permitting expression of LIN-14 protein harboring a small in-frame deletion, which may be largely functional. Therefore, *lin-14(dp69)* is unlikely to be a null allele.

A putative *lin-14* null allele causes precocious dauer arrest, is not dauer defective, and prevents *lin-14* RNAi from suppressing *eak-7;akt-1* dauer arrest

Loss-of-function mutations in *lin-14* have been previously isolated in screens for mutants with precocious developmental phenotypes. LIN-14 is broadly expressed, and *lin-14* loss-of-function mutants have precocious phenotypes in multiple tissues and cell types (Ambros & Horvitz, 1987; Ruvkun & Giusto, 1989). The *lin-14(n536n540)* mutant was isolated in a forward genetic screen for suppression of the *lin-14* gain-of-function phenotypes caused by a deletion of the *lin-4* binding site *lin-14* 3' UTR (*lin-14(n536)*) (Figure 2.1) (Ambros & Horvitz, 1987). In *lin-14(n536n540) lin-14(n536)* gain-of-function phenotypes are entirely suppressed by an early nonsense mutation [*lin-14(n540)*] in exon eight that affects all *lin-14* isoforms (Figure 2.1) (Ambros & Horvitz, 1987; Reinhart & Ruvkun, 2001). *lin-14(n536n540)* is believed to be a null allele based on phenocopying a deficiency that deletes the *lin-14*, locus, and eliminates detectable LIN-14 protein (Ambros & Horvitz, 1987; Ruvkun et al., 1989).

daf-7(e1372);lin-14(n536n540) mutants have been reported to undergo precocious dauer arrest at the L2 larval stage; however, *lin-14(n536n540)* does not have a previously

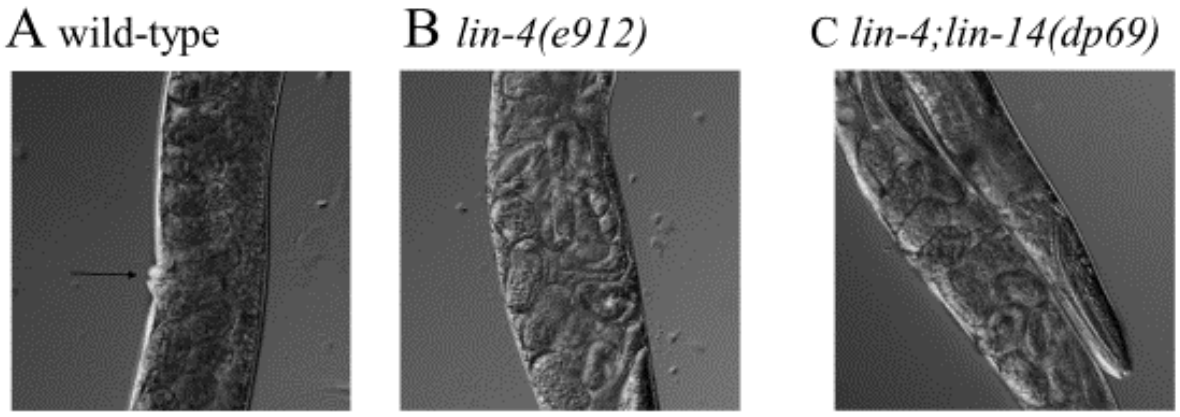
reported Daf-d phenotype (Liu & Ambros, 1989). We generated *eak-7;akt-1;lin-14(n536n540)*, *daf-2(e1368);lin-14(n536n540)*, and *daf-7(e1372);lin-14(n536n540)* mutants. Consistent with the published report, we observed that *daf-7(e1372);lin-14(n536n540)* animals formed small, partially remodeled, dauer larvae at 25°C, without any suppression of the *daf-7(e1372)* Daf-c phenotype (Figure 2.2 panel G, Table 2.2) (Liu & Ambros, 1989). We also found that *daf-2(e1368);lin-14(n536n540)*, and *eak-7;akt-1;lin-14(n536n540)* mutants arrested as dauer larvae precociously without any suppression of Daf-c phenotypes (Figure 2.1 panel E-F, Table 2.2).

In contrast to *lin-14(n536n540)*, dauer entry in *daf-2(e1368);lin-14(dp69)*, *daf-7(e1372);lin-14(dp69)*, *eak-7;akt-1;lin-14(dp69)* animals cultured on *E.coli* strain HT115, as well as in *daf-2(e1368)* and *daf-7(e1372)* animals treated with *lin-14* RNAi appeared to occur normally at the L3 larval stage, based on size and morphology of observed animals (Table 2.2). Treatment with *lin-14 RNAi* did not affect dauer entry in *daf-2(e1368);lin-14(n536n540)* or *daf-7(e1372);lin-14(n536n540)* animals, suggesting that *lin-14* RNAi does not suppress dauer arrest in the absence of *lin-14* gene products. We did observe small numbers of precocious dauer larvae in Daf-c strains treated with *lin-14* RNAi, in both wild-type and *lin-14(dp69)* backgrounds, consistent with a model in which *lin-14* RNAi causes a less penetrant reduction in LIN-14 activity than the strong *lin-14* loss of function mutation *lin-14(n536n540)* (Table 2.2.).

lin-14(dp69) does not have visible heterochronic phenotypes and does not suppress the overt phenotypes of *lin-4* mutants

lin-14(n536n540) mutants have gross visible phenotypes including reduced body size, protruding vulva, defective egg laying, and retention of eggs in the gonad. *lin-4* loss-of-function and *lin-14* gain-of-function animals are elongated, transparent, do not form adult alae, and do not form a functioning vulva, causing them to die early in adulthood due to internal hatching of progeny (Ambros & Horvitz, 1987; Chalfie et al., 1981). *lin-14(dp69)* adults do not have obvious *lin-14* loss-of-function or gain-of-function phenotypes (data not shown). Strong *lin-14* loss-of-function mutations are epistatic to *lin-4* heterochronic phenotypes (Ambros & Horvitz, 1987). *lin-14(dp69)* did not suppress visible phenotypes of *lin-4* mutants (Figure 2.3). Animals exposed to *lin-14* RNAi from egg to adulthood, as was the case in our dauer assays, did not have visible heterochronic phenotypes in adulthood, however animals treated with *lin-14* RNAi for multiple generations has phenotypes including reduced size and protruding vulva that resembled *lin-14* loss-of-function mutants (data not shown). These observations are consistent with models in which *lin-14(dp69)* and *lin-14* RNAi have weaker loss of function phenotypes than *lin-14* alleles isolated from screens for heterochronic phenotypes, and models in which *lin-14(dp69)* has more complex effects on LIN-14 activity.

Figure 2.3. *lin-14(dp69)* does not rescue *lin-4(e912)* phenotypes
Figure 2.3. A. Gonad of wild-type animal. Arrow points to vulva. B. *lin-4(e912)* mutant with accumulation of eggs in the gonad and no vulva. C *lin-4(e912);lin-14(dp69)* compound mutant with accumulation of eggs in the gonad and no vulva.



Discussion

Despite thorough genetic characterization of genes that regulate the dauer decision and extensive study of the heterochronic pathway, the mechanisms by which these networks interact to govern dauer life history-specific developmental events remain poorly understood.

The heterochronic transcription factor LIN-14 is an excellent candidate for a gene that might control the developmental stage that is permissive for animals to commit to the dauer life history. LIN-14 is expressed in fasted L1 larva and is downregulated upon feeding by expression of the *lin-4* microRNA which binds directly to its 3'UTR and triggers degradation of the LIN-14 transcript (Bagga et al., 2005; Ha et al., 1996; Ruvkun et al., 1989; Ruvkun & Giusto, 1989; Wightman et al., 1993). LIN-14 protein levels fall throughout the L1 larval stage (Ruvkun et al., 1989). LIN-14 does not bear obvious sequence similarity to any known genes outside of the nematode family; however, it has been biochemically characterized as a transcription factor which binds to a core GAACRY motif and regulates developmental stage-specific expression of target genes *in vivo* (Hristova et al., 2005). In addition to causing precocious expression of developmental programs specific to L2 and later stages during reproductive development, *lin-14* loss of function mutants have also been reported to undergo precocious dauer arrest at the L2 larval stage, while mutations which cause constitutive expression of

LIN-14 are dauer-defective (Liu & Ambros, 1989). Taken together, these data suggest that LIN-14 activity prevents precocious commitment to the dauer developmental program at the L2 larval stage and is consistent with a model where declining levels of LIN-14 protein, relative to those present in early L1 larva, permit expression of the dauer larval developmental program.

We performed a screen for mutations that suppress the 25°C dauer formation constitutive (Daf-c) phenotype of *eak-7;akt-1* double mutants. The *seak* screen has identified mutations that control dauer arrest by regulating components of the insulin signaling pathway, but that did not emerge from previous screens for suppressors of *daf-2(e1370)* dauer arrest, suggesting that the *eak-7;akt-1* dauer arrest phenotype is sensitive to suppression by mutations that modulate the activity of dauer regulatory pathways but do not cause large enough effects to suppress the dauer constitutive phenotypes of more highly penetrant Daf-c mutations (Delaney et al., 2017; Dumas et al., 2013).

The *seak* screen identified a novel Daf-d mutation in *lin-14, dp69,* that robustly suppressed the Daf-c phenotype of *eak-7;akt-1* mutants. Treatment of *eak-7;akt-1* animals with *lin-14* RNAi phenocopied the *lin-14(dp69)* mutation, initially suggesting that the phenotype of *eak-7;akt-1;lin-14(dp69)* mutants is caused by *lin-14(dp69)* rather than a closely linked background mutation which was not identified by whole genome sequencing, and that *lin-14(dp69)* is a reduction-of-function allele. We observed that the Daf-c phenotypes of *daf-2(e1368)* animals were suppressed when these mutations were placed in a *lin-14(dp69)* background (*daf-2(e1368);lin-14(dp69)*) and treated with *lin-14*

RNAi. Similarly, the Daf-c phenotype of *daf-7(e1372)* animals was also suppressed when they were placed on a *lin-14(dp69)* background and treated with *lin-14* RNAi. The Daf-c phenotypes of *eak-7;akt-1* animals, *daf-2(e1368)* animals, and *daf-7(e1372)* animals were not suppressed by the addition of a putative *lin-14* null allele *lin-14(n536n540)*. An important caveat is that *lin-14(n536n540)* emerged from a forward genetic screen for suppression of *lin-14* gain-of-function phenotypes caused by a deletion in the *lin-14* 3'UTR that prevents *lin-4* binding (*lin-14(n536)*). *lin-14(n536n540)* is a complex allele harboring both the 3'UTR deletion and a premature stop codon in the eighth exon of *lin-14* suggesting that a truncated LIN-14 protein could be made (Ambros & Horvitz, 1987). While it has been reported that the *lin-14(n536n540)* heterochronic phenotypes closely resemble those of a deficiency that completely eliminates the *lin-14* locus, it was not possible to observe a sufficient number of animals carrying *lin-14(n536n540) in trans* to a deficiency to determine whether it rescued any null phenotypes, due to technical reasons likely relating to the pleiotropic phenotypes of these mutants (Ambros & Horvitz, 1987). Thus, it is possible that *lin-14(n536n540)* is not a true null allele. The dauer phenotypes of deficiencies eliminating the *lin-14* locus have not been reported. Furthermore the dauer larvae that are present in *lin-14* loss-of-function mutants develop abnormally. While they are SDS resistant and have dauer alae *lin-14(lf)* dauers arrest after the first larval molt rather than after the second and arrest as partially remodeled dauer larvae that appear to have some dauer cell fates and some non-dauer cell fates within the same animal, suggesting that interactions between *lin-14* null alleles and dauer arrest are complex and that *lin-14* function may be required for dauer development in some tissues(Liu & Ambros, 1989). Nonetheless the fact that we could

not reproduce the *lin-14(dp69)* phenotype with an independent putative *lin-14* null allele precludes any formal genetic epistasis analysis to place the *lin-14* locus into known genetic pathways regulating the dauer decision. It is formally possible that the dauer suppression observed in *lin-14(dp69)* is caused by a closely linked background mutation while the dauer suppression caused by *lin-14* RNAi is due to an off-target effect; however, the observation that *lin-14(dp69)* and *lin-14* RNAi additively suppress the Daf-c phenotypes of *daf-2(e1368)* mutants, while *lin-14* RNAi is unable to suppress the Daf-c phenotypes of *daf-2(e1368);lin-14(n536n540)* compound mutants seems more consistent with models in which *lin-14(dp69)* and *lin-14* RNAi prevent dauer arrest by modulating *lin-14* activity in some way. Our data could be consistent with multiple models in which non-null changes in *lin-14* activity modulate dauer entry.

The *lin-14(dp69)* mutation causes penetrant suppression of *eak-7;akt-1* dauer arrest when animals are cultured on the *E. coli* strain OP50, but not when animals are cultured on the *E.coli* strain HT115. Levels of LIN-14 protein are regulated by feeding, so it is possible that culture on different bacterial strains affects dauer by regulating LIN-14 (Ruvkun & Giusto, 1989). However, culture on HT115 might increase dauer arrest in sensitized contexts by upregulating DAF-16 activity, or in parallel to both LIN-14 and DAF-16. Culture on HT115 alters DAF-16-dependent lifespan phenotypes, suggesting food type may affect DAF-16 activity (Amrit et al., 2016; Chen et al., 2015) . The hypothesis that culture on HT115 generally enhances the Daf-c phenotypes of insulin signaling mutants could be tested by performing dauer assays with Daf-c mutants on HT115 and OP50 at equal optical densities under sensitized conditions, for example in synchronized cultures of *daf-2/*IGFr mutants at 22.5°C (Gems et al., 1998; Patel et al., 2008). In several *daf-2*

alleles (including *daf-2(m120)* and *daf-2(m596)*) synchronized populations include both animals that arrest as dauers and animals that undergo reproductive development when animals are cultured at 22.5°C when on OP50 (Gems et al., 1998). If culture on HT115 enhances dauer arrest independently of *lin-14* we would expect to see an increased percentage of animals arresting as dauers when cultured on HT115 under these conditions (Gems et al., 1998). We could potentially do similar experiments with *eak-7;akt-1* animals or *eak-7* and *akt-1* mutants individually to determine whether HT115 affects dauer entry in these contexts specifically; however, appropriate sensitized conditions in which we would expect to observe a significant proportion of dauer and non-dauer animals within a population have not been reported for these strains. These experiments could also be done in parallel with strains harboring *daf-2;lin-14(dp69)* mutations, *eak-7;akt-1;lin-14(dp69)* mutations and *eak-7;lin-14(dp69)* and *akt-1;lin-14(dp69)* mutations. If increased dauer arrest on HT115 is specific to *lin-14(dp69)* mutants then we would expect to see increased dauer arrest in strains harboring *lin-14(dp69)* mutations only. The observation that *lin-14* RNAi suppresses the *eak-7;akt-1* Daf-c phenotype, despite culture on HT115, and the observation that the combination of *lin-14(dp69)* and *lin-14* RNAi cause penetrant dauer suppression phenotypes suggest that changes in LIN-14 activity may be able to influence the dauer decision independently of food source.

All *lin-14* transcripts sequenced from *lin-14(dp69)* mutants contained either frameshifts or small in-frame deletions, though it is possible that there is a small population of *lin-14* transcripts with wild-type splice-junction sequences between exons five and six that we were not able to identify given the limited number of transcripts we sequenced. We do not observe the severe pleiotropic *lin-14* loss-of-function phenotypes that are present in

the predicted null mutant *lin-14(n536n540)* in *lin-14(dp69)*. Unlike known *lin-14* loss-of-function alleles, *lin-14(dp69)* does not suppress *lin-4(e912)* mutant phenotypes and does not cause arrest as a partially remodeled dauer larvae after the first larval molt rather than after the second (Figure 2.3, Table 2.2). These observations, together with the reported observation that the phenotypes of *lin-14(n536n540)* closely mirror the phenotypes of a deficiency fully eliminating the *lin-14* locus, suggest that *lin-14(dp69)* is not a stronger loss of function allele that *lin-14(n536n540).*

The observation that *lin-14(dp69)* animals do not have obvious *lin-14* loss-of-function phenotypes could be consistent with a model in which the LIN-14 protein encoded by transcripts harboring the in-frame deletion is able to perform some developmental functions of wild-type LIN-14. However, the deletion of nine amino acids and the P-A conversion in the in-frame *lin-14* transcripts we identified could affect the function of the LIN-14 protein. The missing amino acids might, for example, alter the binding of LIN-14 to co-regulators, resulting in different patterns of expression or localization and changes in its downstream effects on transcription (Hristova et al., 2005). Thus, the differences in phenotypes caused by *lin-14(dp69)* and *lin-14(n536n540)* could be due to neomorphic (change-of-function) effects on the LIN-14 protein caused by *lin-14(dp69).* Since some tissues are more sensitive to RNAi than others, treating *lin-14(dp69)* with *lin-14* RNAi might exacerbate a neomorphic effect by further altering the tissue distribution of LIN-14 protein, resulting in dauer regulatory phenotypes unrelated to those of *lin-14* null alleles. For example, it is possible that LIN-14 activity in neurons but not other tissues could suppress dauer arrest without visible heterochronic phenotypes, and an increase in neuronal activity of LIN-14 caused by *lin-14(dp69)* could cause an increase in

neuronal LIN-14 expression, while *lin-14* RNAi contributes to the phenotype by reducing LIN-14 levels in non-neuronal tissues. We could test the hypothesis that the molecular lesion present in *lin-14(dp69)* animals causes changes in LIN-14 protein expression levels or localization by engineering animals expressing a single copy LIN-14::GFP fusion, and an identical mutation to *lin-14(dp69)* using CRISPR-CAS9. Single-copy LIN-14::GFP fusions have previously been generated and are amenable to examination of GFP expression by microscopy (Arribere et al., 2014). The effects of the *lin-14(dp69)* mutation on LIN-14 mediated transcriptional events could be determined using RNA-seq of wild-type, *lin-14(dp69)*, *eak-7;akt-1*, and *eak-7;akt-1;lin-14(dp69)* animals at L1 and L2 or L2d larval stages, or in a more limited manner by using qPCR of previously identified LIN-14 targets.

We sequenced the splice junction containing *lin-14(dp69)* using two unique primers complementary to the *lin-14a* specific and *lin-14b* specific exons and did not detect evidence of isoform-specific differences in splicing events (Table 2.1). RNA that is available to be translated. This could reduce levels of specific *lin-14* transcripts or of all *lin-14* transcripts. The hypothesis that *lin-14(dp69)* causes reductions in *lin-14* transcript levels is consistent with the observation that *lin-14* RNAi phenocopies *lin-14(dp69)* suppression of *eak-7;akt-1* dauer arrest. An additive reduction in levels of some *lin-14* transcripts caused by *lin-14(dp69)* and *lin-14* RNAi could also be consistent with the observation that *lin-14* RNAi and *lin-14(dp69)* additively suppress the Daf-c phenotypes of *daf-2(1368)* and *daf-7(e1372)* mutants. *lin-14* isoforms have independently mutable functions with *lin-14a* being required during the early L1 larval stage to prevent precocious execution of L2 specific developmental programs, while *lin-14b* is required

late in the L1 larval phase to prevent precocious expression of L3 specific programs during the L2 larval stage (Ambros & Horvitz, 1987; Reinhart & Ruvkun, 2001). Given the similarity between *lin-14a* phenotypes and *lin-14* null phenotypes, it seems unlikely that *lin-14(dp69)* or *lin-14* RNAi are specifically affecting *lin-14a*. Our data could be consistent with a model in which *lin-14(dp69)* and *lin-14* RNAi affect the *lin-14b* isoform. Importantly, the L2 larval stage-specific patterning events that are lost in *lin-14b* mutants do occur, albeit precociously, in *lin-14* null mutants; thus, if *lin-14* RNAi and *lin-14(dp69)* caused suppression of dauer arrest through a loss of function in the *lin-14b* genetic activity it could explain why the dauer suppression observed in these contexts is not observed in putative *lin-14* null alleles. *lin-14b* affects the second larval stage, and loss of *lin-14b* could prevent L2d specific events such as accumulation of dafachronic acids that are critical for dauer entry (Ambros & Horvitz, 1987; Golden & Riddle, 1984; Reinhart & Ruvkun, 2001; Schaedel et al., 2012). A molecular analysis of *lin-14* isoforms demonstrated that increased *lin-14a* expression could rescue *lin-14b* phenotypes (Reinhart & Ruvkun, 2001). The authors hypothesized that the proteins encoded by the two *lin-14* isoforms do not have distinct molecular functions, but that *lin-14b* is required to maintain a critical concentration of LIN-14 protein late in the L1 larval stage (when LIN-14 protein levels are falling due to interaction with *lin-4*) that is required for L2 specific developmental events (Reinhart & Ruvkun, 2001). An intervention, such as *lin-14* RNAi, that slightly reduces *lin-14* transcript levels, might produce a phenotype similar to a *lin-14b* mutant by allowing LIN-14 levels to fall below a critical threshold late in the L1 larval stage, and therefore cause events that occur during the L2 or L2d larval stage to be skipped, resulting in phenotypes distinct from those

observed in *lin-14(n536n540)* mutants in which L2-specific developmental events occur early. Mutations that specifically affect *lin-14b* isoforms have not been tested for effects on dauer arrest. We could test the hypothesis that *lin-14b* alleles have Daf-c phenotypes by obtain existing *lin-14b* mutants and crossing them into Daf-c backgrounds, or by generating *lin-14b* mutants using CRISPR/Cas9-based gene editing and testing these mutants for their ability to suppress dauer arrest.

Published work has revealed roles for two other regulators of developmental timing, *hbl-1*/hunchback and *lin-42*/period, in regulating dauer formation constitutive and defective phenotypes. LIN-42/per is a heterochronic transcription factor that, like DAF-12, is important for hypodermal patterning during the L3 larval stage and proper development of the somatic gonad (Tennessen et al., 2006; Tennessen et al., 2010). *lin-42* mutants have a Daf-c phenotype when cultured at 27°C, and LIN-42 overexpression at L2 suppresses the Daf-c phenotypes of insulin signaling, TGF-β, dafachronic acid pathway, and *daf-12(rh273)* ligand-binding-domain mutants (Tennessen et al., 2010). LIN-42 protein directly was identified as a direct binding partner of DAF-12 by yeast two-hybrid, suggesting that that LIN-42 may regulate DAF-12 transcriptional activity directly (Tennessen et al., 2010). LIN-42/Per has a dynamic expression pattern; high levels of LIN-42 protein are present during L1 and L2 intermolt periods, while low levels of LIN-42 protein are detected near molts. Falling levels of LIN-42 at the L1 to L2d and L2d to dauer molts might permit dauer arrest by permitting activity of DAF-12.

The hunchback-like transcription factor HBL-1, is involved in promoting L2-specific developmental programs and inhibiting L3 developmental programs (Abrahante et al.,

2003). Two independent loss-of-function alleles in *hbl-1* partially suppress dauer constitutive phenotypes of *daf-12(rh273)* ligand-binding-domain mutants and *daf-9*/cytochromeP450 dafachronic acid biosynthesis mutants (Karp & Ambros, 2011). This suggests that *hbl-1* promotes dauer entry, either through ligand-independent regulation of DAF-12, or through regulation of shared *daf-12-hbl-1* target genes. Interestingly the same *hbl-1* loss-of-function mutations cause enhanced dauer arrest in *daf-2*/IGFr and *daf-7*/TGF-β mutants, through a mechanism that does not require either *daf-16* (in the context of *daf-7* mutation) or *daf-5* (in the context of *daf-2* mutation) suggesting that HBL-1 acts independently of the insulin and TGF-β pathways (Karp & Ambros, 2011). HBL-1::GFP transgenes regulated by the endogenous *hbl-1* promoter and 3'UTR are highly expressed during the L1 larval stage, with expression declining during L2d and becoming undetectable in dauer larvae (Karp & Ambros, 2011). The authors propose a model in which decreasing levels of HBL-1 protein during the L2d larval stage result in two separable, stage-specific HBL-1 activities that affect the dauer decision. During the L1 larval stage high levels of HBL-1 inhibit dauer formation, possibly by negatively regulating DAF-12/NHR in parallel to the insulin signaling and TGF-β pathways. In contrast, the lower levels of HBL-1 present in the L2d stage coordinate with DAF-12 to promote dauer formation (Karp & Ambros, 2011).

As a regulator of L1 specific events, LIN-14 activity occurs prior to activity of LIN-42 and HBL-1 in the heterochronic pathway. Events that occur at the L2d larval stage, such as dafachronic acid regulation, are critical for dauer entry; HBL-1 and LIN-42 might act at the L2 larval stage to regulate the timing of these processes. It is unclear how L2d specific events that regulate the dauer decision occur in LIN-14 null mutants that arrest as

dauers after a single larval molt, or how LIN-42 and HBL-1 activities might modulate these events in this context. In *lin-14(dp69)* mutants and in strains treated with *lin-14* RNAi, dauer entry occurs after the second larval molt. These perturbations in LIN-14 activity might modulate L2 specific events, such as HBL-1 and LIN-42 expression, in a manner that is distinct from *lin-14* null alleles. For example, *lin-14(dp69)* and *lin-14* RNAi could cause reduction in the *lin-14b* genetic activity, which affects L2 specific patterning events and could affect HBL-1 or LIN-42 expression or dafachronic acid accumulation during the L2d larval stage. For example, loss of the *lin-14b* activity could cause precocious LIN-42 expression at the L1 to L2 transition. Experiments in which constitutive expression of LIN-42 at the L2d larval stage prevented dauer arrest suggest that this change might be sufficient to explain the Daf-d phenotypes observed in *lin-14(dp69)* and on *lin-14* RNAi (Tennessen et al., 2010). We could test this model by determining whether *lin-14* RNAi or *lin-14(dp69)* suppressed the Daf-c phenotypes observed in *lin-42* loss of function mutants; if *lin-42* activity regulates the dauer decision downstream of *lin-14* we would expect *lin-14(dp69)* and *lin-14* RNAi to be unable to suppress the Daf-c phenotypes of *lin-42* mutants. Since *lin-14(dp69)* and *lin-14* RNAi are not null alleles, we cannot do formal epistasis analysis using them, and it would be important to test any hypothesis we formed with genetic experiments by collecting molecular data. We could, for example, determine whether *lin-14(dp69)* or *lin-14* RNAi alters the expression of a LIN-42::GFP transgene. In contrast we might expect LIN-42 activity to be absent after the first larval molt in *lin-14(n536n540)* mutants in Daf-c contexts since these animals have entered dauer after the first larval molt and LIN-42 expression is downregulated in dauer larvae (Tennessen et al., 2010).

In conclusion, we were unable to establish a convincing model for how *lin-14(dp69)* affects LIN-14 activity. *lin-14(dp69)* mutants lack obvious *lin-14* loss-of-function and *lin-14* gain-of-function phenotypes. We cannot rule out the possibility that *lin-14(dp69)* causes gain-of-function or neomorphic effects. In dauer-constitutive backgrounds, strong *lin-14* loss-of-function mutants enter a dauer-like diapause after the first larval molt, suggesting that regulation of the dauer decision is not a primary activity of *lin-14* (Liu & Ambros, 1989). *lin-14b* mutations affect developmental events specific to the L2 larval stage in ways that are distinct from *lin-14* null phenotypes. If *lin-14(dp69)* or *lin-14* RNAi affect *lin-14b* this could explain why these interventions suppress dauer arrest while a putative *lin-14* null allele does not (Ambros & Horvitz, 1987; Reinhart & Ruvkun, 2001). While *lin-14(n536n540)* mutants enter dauer after the first larval molt, *lin-14(dp69)* mutants and animals treated with *lin-14* RNAi appear to enter dauer after the second larval molt. This suggests that they could suppress dauer arrest by regulating L2d specific events, consistent with these interventions affecting *lin-14b* activity. Our data are consistent with multiple models in which changes in *lin-14* function might affect dauer entry, including via isoform-specific or neomorphic effects. Numerous additional experiments would be required to differentiate between these models.

More broadly, our data are consistent with other published reports showing that other genes which regulate L2 and L3 cell fates, including the hunchback-like transcription factor *hbl-1* and the period homolog *lin-42*, can influence the dauer decision, by acting downstream of or in parallel to insulin signaling and TGF-β signaling. Further studies of these pathways may help us to achieve a detailed mechanistic understanding of the interactions between signaling pathways that influence the dauer decision and genes that

coordinate stage specific developmental programs. This may in turn reveal conserved mechanisms underlying the coordination between developmental timing and plasticity, which are necessary for robust development in changing environmental conditions.

Table 2.1. Splice junctions between exons affected by *lin-14(dp69)* in wild-type and *lin-14(dp69)* mutant transcripts

Table 2.1. Splice junctions affected by *lin-14(dp69)* in wild-type and *lin-14(dp69)* mutant transcripts. Number of TOPO cloned LIN-14 cDNAs sequenced with each observed splice junction is shown.

Sequence of junction between exons 5 and 6	Predicted effect on protein	Number of reads containing junction: LIN-14B from wild-type	Number of reads containing junction:LIN-14A from wild-type	Number of reads containing junction: LIN-14B from *lin-14(dp69)*	Number of reads containing junction: LIN-14A from *lin-14(dp69)*
TCAC-CCGC	None: expected WT splice junction.	1	5	0	0
TACG-CCGC	5 amino acid deletion and frameshift.	0	0	14	9
CGG-CCGC	In frame 9 amino acid deletion and P to A conversion.	0	0	1	2

Table 2.2. Effects of *lin-14* mutations and *lin-14* RNAi on dauer entry

Bacterial strain, shown in figure	Genotype	Non dauer	L3 dauer	Precocious L2 dauer	Percent dauer	Standard deviation 3 plates	Strain compared to	P (T-test)	Change in percent dauer
OP50	WT	241	0	0	0	0			
	lin-14(dp69)	233	0	0	0	0			
shown	*eak-7(tm3188);akt-1(ok525)*	6	264	0	97.77777778	3.120812			
	eak-7;akt-1;lin-14	276	37	0	11.82108626	6.990499	*eak-7;akt-1*	0.0015946	-85.95669
OP50	WT	169	0	0	0	0			
	lin-14(dp69)	324	0	0	0	0			
	eak-7(tm3188);akt-1(ok525)	21	346	0	94.27792916	3.768534			
	eak-7;akt-1;lin-14	254	47	0	15.61461794	38.33195	*eak-7;akt-1*	0.098483	-78.66331
OP50	WT	319	0	0	0	0			
	lin-14(dp69)	251	0	0	0	0			
	eak-7(tm3188);akt-1(ok525)	8	200	0	96.15384615	2.663328			
	eak-7;akt-1;lin-14	197	13	0	6.19047619	3.60957	*eak-7;akt-1*	0.0006603	-89.96337
OP50	*eak-7(tm3188);akt-1(ok525)*	23	137	0	85.625	8.499993			
HT115	*eak-7(tm3188);akt-1(ok525)*	1	112	0	99.11504425	1.480385			
OP50	*eak-7;akt-1;lin-14(dp69)*	214	16	0	6.956521739	5.286488	*eak-7;akt-1* OP50	0.0020077	-78.66848
HT115	*eak-7;akt-1;lin-14(dp69)*	218	113	0	34.13897281	11.98634	*eak-7;akt-1;lin-14* OP50	0.0480661	27.182451
OP50	*eak-7;akt-1;lin-14(dp69)*	182	5	0	2.673796791	5.891329			
HT115	*eak-7;akt-1;lin-14(dp69)*	56	266	0	82.60869565	16.7488	*eak-7;akt-1;lin-14* OP50	0.0012005	79.934899
HT115	*eak-7(tm3188);akt-1(ok525)*	0	290	0	100	0			
	eak-7;akt-1 + lin-14 RNAi	105	81	0	43.5483871	2.012844	*eak-7;akt-1*	0.0004241	-56.45161
	eak-7;akt-1;lin-14(dp69)	67	163	0	70.86956522	17.4131	*eak-7;akt-1*	0.1223459	-29.13043
	eak-7;akt-1;lin-14 + *lin-14* RNAi	232	1	0	0.429184549	0.648708	*eak-7;akt-1 +lin-14* RNAi	0.0012608	-43.1192
HT115	*eak-7(tm3188);akt-1(ok525)*	13	462	0	97.26315789	0.399157			
	eak-7;akt-1+ lin-14 RNAi	445	110	2	20.10771993	5.490408	*eak-7;akt-1*	0.001801	-77.15544
	eak-7;akt-1;lin-14(dp69)	107	223	0	67.57575758	10.68594	*eak-7;akt-1*	0.0467458	-29.6874
	eak-7;akt-1;lin-14 + *lin-14* RNAi	322	0	5	1.529051988	1.366883	*eak-7;akt-1* + *lin-14* RNAi	0.0271358	-18.57867
	WT	294	0	0	0	0			
HT115	*eak-7(tm3188);akt-1(ok525)*	19	217	0	91.94915254	2.554622			
shown	*eak-7;akt-1+ lin-14 RNAi*	224	3	0	1.321585903	1.882664	*eak-7;akt-1*	5.248E-05	-90.62757
	eak-7;akt-1;lin-14(dp69)	145	166	0	53.37620579	35.82248	*eak-7;akt-1*	0.2293835	-38.57295
	eak-7;akt-1;lin-14 + *lin-14* RNAi	294	0	8	2.649006623	2.380978	*eak-7;akt-1* + *lin-14* RNAi	0.6396678	1.3274207
	WT	198	0	0	0	0			
HT115	*eak-7(tm3188);akt-1(ok525)*	32	269	0	89.36877076	2.650669			
	eak-7;akt-1;lin-14(n536n540)	33	0	234	87.64044944	16.07977	*eak-7;akt-1*	0.5388247	-1.728321
	WT	119	0	0	0	0			
HT115	*eak-7(tm3188);akt-1(ok525)*	0	290	0	100	0			
	eak-7;akt-1;lin-14(n536n540)	0	0	54	100	0		NA	0
	eak-7;akt-1;lin-14 +*lin-14* RNAi	0	0	16	100	0		NA	0
HT115	*eak-7(tm3188);akt-1(ok525)*	14	236	0	94.4	1.998412			
shown	*eak-7;akt-1;lin-14(n536n540)*	2	0	154	98.71794872	1.268902	*eak-7;akt-1*	0.0436531	4.3179487
	eak-7;akt-1;lin-14 +*lin-14* RNAi	0	0	123	100	0	*eak-7;akt-1*	0.0389833	5.6
	WT	335	0	0	0	0			
Ht115	*WT*	125	0	0	0	0			
	lin-14(dp69)	198	0	0	0	0			
	daf-2(e1368)	11	256	0	95.88014981	2.524053			
	daf-2 +lin-14 RNAi	93	251	170	81.90661479	7.925754	*daf-2*	0.0720381	-13.97354
	daf-2;lin-14(dp69)	12	316	0	96.34146341	3.538931	*daf-2*	0.8037912	0.4613136
	daf-2;lin-14(n536n540)	4	0	272	98.55072464	1.519912	*daf-2*	0.2805863	2.6705748
	daf-2;lin-14(n536n540) + *lin-14* RNAi	12	0	617	98.09220986	0.581193	*daf-2*	0.3694737	2.21206
	daf-2;lin-14(dp69) + *lin-14* RNAi	291	0	43	12.8742515	6.978688	*daf-2*	0.0030769	-83.0059
Ht115	*WT*	151	0	0	0	0			
	lin-14(dp69)	204	0	0	0	0			
shown	*daf-2(e1368)*	5	296	0	98.33887043	1.703265			
	daf-2 +lin-14 RNAi	37	129	0	77.71084337	17.39518	*daf-2*	0.1413892	-20.62803
	daf-2;lin-14(dp69)	13	329	0	96.19883041	1.322757	*daf-2*	0.3420006	-2.14004
	daf-2;lin-14(n536n540)	4	0	121	96.8	2.01567	*daf-2*	0.4796457	-1.53887
	daf-2;lin-14(n536n540) + *lin-14* RNAi	2	0	258	99.23076923	1.012895	*daf-2*	0.0830394	0.8918988
	daf-2;lin-14(dp69) + *lin-14* RNAi	435	0	42	8.805031447	5.9536	*daf-2*	0.0021943	-89.53384
HT115	WT	335	0	0	0	0			
	lin-14(dp69)	254	0	0	0	0			
	daf-2(e1368)	17	337	0	95.19774011	1.780897			
shown	*daf-2 +lin-14 RNAi*	55	208	0	79.08745247	13.19102	*daf-2*	0.036376	-16.11029
	daf-2;lin-14(dp69)	18	303	0	94.39252336	2.770984	*daf-2*	0.7378847	-0.805217
	daf-2;lin-14(n536n540)	7	0	303	97.74193548	2.398801	*daf-2*	0.2414556	2.5441954
	daf-2;lin-14(n536n540) + *lin-14* RNAi	0	0	184	100	0	*daf-2*	0.0384397	4.8022599
	daf-2;lin-14(dp69) + *lin-14* RNAi	323	1	0	0.308641975	0.54467	*daf-2*	8.86E-05	-94.8891

Bacterial strain, shown in figure	Genotype	Non dauer	L3 dauer	Precocious L2 dauer	Percent dauer	Standard deviation 3 plates	Strain compared to	P (T-test)	Change in percent dauer
HT115	*daf-7(e1372)*	0	168	0	100	0			
	daf-7 + lin-14 RNAi	16	680	0	97.70114943	1.456355	*daf-7*	0.196075	-2.298851
	daf-7 + daf-12 RNAi	199	9	0	4.326923077	5.316376	*daf-7*	0.0010334	-95.67308
Ht115	*daf-7(e1372)*	6	202	0	97.11538462	8.925284			
	daf-7(e1372) + lin-14 RNAi	21	212	0	90.98712446	11.47177	*daf-7*	0.7762961	-6.12826
	daf-7(e1372) + daf-12 RNAi	183	5	0	2.659574468	1.628045	*daf-7*	0.0027697	-94.45581
HT115	*daf-7(e1372)*	0	80	0	100	0			
	daf-7(e1372) + lin-14 RNAi	5	97	0	95.09803922	0.935772	*daf-7*	0.0123681	-4.901961
	daf-7(e1372);lin-14(dp69)	20	59	0	74.6835443	3.517741	*daf-7*	0.3193276	-25.31646
	daf-7(e1372) + daf-12 RNAi	122	4	0	3.174603175	18.59834	*daf-7*	0.0004402	-96.8254
shown	*daf-7;lin-14 + lin-14 RNAi*	105	37	0	26.05633803	3.259852	*daf-7*	0.000649	-73.94366
HT115	WT	317	0	0	0	0			
	lin-14(n536n540)	16	0	0	0	0			
	daf-7(e1372)	1	133	0	99.25373134	1.202813			
	daf-7;lin14	28	0	151	84.3575419	3.499091	*daf-7*	0.0101516	-14.89619
HT115	WT	208	0	0	0	0			
	lin-14(n536n540)	12	0	0	0	0			
	daf-7(e1372)	1	380	0	99.73753281	0.246731			
	daf-7;lin14	9	0	72	88.88888889	3.227424	*daf-7*	0.0207361	-10.84864
HT115	WT	305	0	0	0	0			
	lin-14(n536n540)	105	0	0	0	0			
	daf-7(e1372)	1	236	0	99.57805907	0.481125			
	daf-7;lin14	1	0	91	98.91304348	4.12393	*daf-7*	0.493443	-0.665016

Table 2.2. Number of animals with each dauer phenotype, percentage of dauer and non-dauer animals, standard deviation calculated based on percentage of animals arresting as dauer larvae on each of three plates, strain compared to for measures of significance, paired two tailed students T-tests, and the change in the percentage of animals undergoing dauer arrest based on the total. Representative experiment plotted in Figure 2.2 is marked "shown". Differences were assessed as significant if $P<.05$, or if $P<0.1$ with a greater than 50% change in the total percentage of animals forming dauer larvae.

Materials and Methods

Strains and reagents

The following mutant alleles were used: *eak-7(tm3188);akt-1(ok525)*, *lin-14(dp69)*, *lin-14(n536n540)*, *lin-4(e912)*, *daf-2(e1368)*, *daf-7(e1372)* (Alam et al., 2010; Ambros & Horvitz, 1987; Chalfie et al., 1981; Dumas et al., 2013; Kimura et al., 1997; Reinhart & Ruvkun, 2001; Ren et al., 1996). *lin-4(e912)* was maintained and crossed by balancing with *dpy-10(e128)*, which is closely linked on chromosome II, and picking heterozygotes which lacked either *lin-4* or *dpy-10* phenotypes: this balanced strain is available from the Caenorhabditis Genetics Center (strain CB2627) (Chalfie et al., 1981). RNAi clones targeting *lin-14* and *daf-12* are from the Ahringer library (Kamath & Ahringer, 2003). Animals were maintained on nematode growth media (NGM) seeded with *E. coli* strain OP50 at 15°C using standard methods, unless otherwise noted. Compound mutants were generated using standard protocols, genotypes were confirmed with PCR-based genotyping assays.

Mapping

The screen for suppressors of *eak-7;akt-1* dauer arrest was described previously (Dumas et al., 2013). *lin-14(dp69)* was isolated from an *eak-7;akt-1;seak* strain designated BQ10. BQ10 was outcrossed to BQ29, a recombinant inbred strain generated by introgression of *eak-7(tm3188)* and *akt-1(ok525)* mutations into the polymorphic CB4856 strain. Single-

nucleotide polymorphism mapping was performed using a set of 48 primer pairs (8 per chromosome) that flank Dra I restriction site polymorphisms. This was sufficient to map the suppression of dauer arrest phenotype to an interval on the X-chromosome containing *lin-14(dp69)* and two other mutations (*dp68*, and *dp70*) identified by whole genome sequencing. BQ10 was then outcrossed 6 times to *eak-7(tm3188);akt-1(ok525)* on the N2 background. The *dp68* and *dp70* mutations were eliminated by outcrossing to *eak-7;akt-1* and using PCR-based genotyping assays specific to the *dp68*, *dp69*, and *dp70* mutations. *eak-7(tm3188);akt-1(ok525);lin-14(dp69)* strains had an identical dauer suppression phenotype to BQ10, while *eak-7;akt-1* strains carrying the *dp68* and *dp70* mutations had Daf-c phenotypes indistinguishable from *eak-7;akt-1*. *lin-14(dp69)* was crossed into an N2 wild-type background, and then crossed into an un-mutagenized *eak-7(tm3188);akt-1(ok525)* strain to eliminate effects of background mutations linked to *eak-7* or *akt-1* in BQ10. Suppression of dauer arrest in *eak-7(tm3188);akt-1(ok525);lin-14(dp69)* animals from this cross compared to *eak-7;akt-1* siblings, was similarly penetrant to the suppression of *eak-7;akt-1* dauer arrest observed in BQ10.

Transcript sequencing

Animals from synchronized egg lays of 100+ gravid hermaphrodites were grown at 25°C for 24 hours to approximately the L2 larval stage. Animals were washed twice in M9 buffer. Total RNA was extracted using TRIzol reagent (Invitrogen) and purified using a RNeasy kit (Qiagen). cDNA was synthesized using a SuperScript III reverse transcriptase kit and random hexamers. Exon-exon junction containing the *lin-14(dp69)* mutation was amplified from cDNA using a standard PCR protocol. Bands

corresponding to *lin-14* transcripts were gel extracted using the QiaQuick gel extraction kit (Qiagen) and cloned using the TOPO XL vector and kit (Invitrogen). Cloned transcripts were Sanger sequenced by the University of Michigan sequencing core.

Dauer assays

Dauer assays were performed as previously described (Dumas et al., 2013). Animals from synchronized six-hour egg lays were cultured at 25°C for 60 hours, and dauer and non-dauer lava were counted under a dissecting microscope. Precocious dauer larva were identified based on reduced body size and incomplete dauer phenotypes, as described (Liu & Ambros, 1989).

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Chapter 3 Mechanisms of longevity extension by FoxO transcription factors

Introduction

Studies of *C. elegans* genetics were initially intended to elucidate the molecular basis of development-the process that turns a single fertilized cell into a complex multi-cellular adult organism. Over the past 30 years, studies of *C. elegans* have also had a substantial impact on understanding the molecular basis of aging-the process which converts young robust healthy animals into old, frail animals with increased probability of morbidity and mortality.

It is common to think of aging as a stochastic process involving increasing risk of separate pathologies-with mechanisms that drive tumorigenesis being distinct from mechanisms causing insulin resistance, atherosclerosis, cataracts, or neurodegenerative disease. However, the conservation of age-related pathologies across vertebrates suggests that aging is coordinately regulated (Miller et al., 2011b; Pitt & Kaeberlein, 2015). Aged mammals develop similar patterns of co-morbidities, but on vastly different time scales: an animal with insulin resistance, cancer, dementia, and cataracts might be a ninety-year-old human, or a twelve-year-old dog. Large differences in species lifespan can evolve in closely related species: a house mouse can live for three years, while a deer mouse can live for eight, and a brown bat for over thirty (Harper et al., 2007). This

suggests that genetic changes can cause parallel delays in multiple age-associated pathologies.

Several interventions are also able to robustly alter the aging process within species. Caloric restriction, a set of interventions that limits nutrient availability without malnutrition, extends lifespan from yeast to primates (Colman et al., 2009; Greer et al., 2007; Kaeberlein et al., 2004; Mattison et al., 2012; McCay et al., 1935). Treatments with drugs including aspirin, rapamycin, acarbose, 17-α-estradiol, and metformin, as well as supplementation with NAD+ precursors, have been reported to extend lifespan in mice, with the first three drugs having done so reproducibly in multi-site trials (Harrison et al., 2014; Harrison et al., 2009; Miller et al., 2011a; Strong et al., 2008; Zhang et al., 2016). Rapamycin also extends lifespan in invertebrates and yeast, suggesting that the mechanisms of longevity determination it affects are ancient and highly conserved (Bjedov et al., 2010; Powers et al., 2006; Robida-Stubbs et al., 2012). These studies suggest that manipulating highly conserved physiological mechanisms can delay multiple age-associated morbidities and mortality.

Emerging evidence suggests that interventions that increase longevity in animal models may be useful to treat age-related morbidities in the clinic. A clinical study has suggested that patients treated with metformin have lower all-cause mortality than age-matched non-diabetic patients (Bannister et al., 2014; Barzilai et al., 2016). Another clinical study has found that treatment with a low-dose analogue of rapamycin can improve some aspects of immune function in elderly patients (Mannick et al., 2014). Further development of interventions that can treat multiple age-related morbidities would be

facilitated by a detailed understanding of the molecular mechanisms which lead from environmental signals to increased longevity.

The FoxO family transcription factor DAF-16 promotes longevity in *C. elegans*

A single gene mutation that controls longevity was first discovered using a forward genetic screen for *C. elegans* strains with increased adult lifespan (Klass, 1983). The longevity phenotype was mapped to a single genetic locus, which was named *age-1* (Friedman & Johnson, 1988; Johnson, 1990). Subsequent studies determined that animals with loss-of-function mutations in the dauer-formation constitutive gene *daf-2* were long lived, and that their long lifespan required the downstream dauer-defective gene *daf-16* (Dorman et al., 1995; Kenyon et al., 1993). Cloning of the affected genes revealed that *daf-2* encodes a receptor tyrosine kinase homologous to mammalian insulin and insulin-like growth factor (IGFr) receptors, *age-1* encodes a phosphoinositide 3-kinase, and *daf-16* encodes a FoxO family transcription factor (Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997). This established insulin and insulin-like-growth-factor signaling (IIS) as the first signal transduction pathway to regulate longevity.

Insulin signaling regulates DAF-16/FoxO activity through conserved mechanisms. Activation of *daf-2*/IGFr leads to phosphoinositide phosphorylation by AGE-1/PI3K. This causes recruitment and activation of PDK-1, which in turn activates the kinases AKT and AKT-2. AKT-1 and AKT-2 phosphorylate DAF-16/FoxO at conserved RxRxxS/T sites (Brunet et al., 1999; Lee et al., 2001; Paradis et al., 1999; Paradis &

Ruvkun, 1998). Phosphorylated DAF-16 is exported from the nucleus and targeted for degradation (Berdichevsky et al., 2006; Brunet et al., 2002; Li et al., 2007). Loss-of-function mutations in *pdk-1*, *akt-1*, and *akt-2* cause DAF-16/FoxO-dependent increases in lifespan (Alam et al., 2010; Paradis et al., 1999; Paradis & Ruvkun, 1998).

DAF-16/FoxO activity is regulated by other kinases in addition to components of the insulin signaling pathway. CST-1/MST-1 phosphorylates DAF-16 and mammalian FoxO3, promoting nuclear translocation of mammalian FoxO3, and CST-1 overexpression extends *C. elegans* lifespan in a DAF-16/FoxO dependent manner (Lehtinen et al., 2006). Similarly, mammalian c-Jun N-terminal kinase (Jnk) phosphorylates and activates FoxO4 and the *C. elegans* homolog JNK-1 promotes longevity in a DAF-16/FoxO dependent manner (Oh et al., 2005). DAF-16/FoxO is also phosphorylated and activated by UNC-43/CAMKII and the phosphate group added by UNC-43 is removed by TAX-6/calcineurin, with UNC-43/CAMKII promoting nuclear accumulation of DAF-16/FoxO and longevity (Tao et al., 2013). Murine FOXO3 can also be phosphorylated and transcriptionally activated or dephosphorylated and inactivated by CAMKII and calcineurin in a conserved manner (Tao et al., 2013).

Interestingly, nuclear localization of DAF-16/FoxO is not sufficient for full activation and lifespan extension. A DAF-16::GFP transgene with all three AKT-1 phosphorylation sites mutated to alanine is localized to the nucleus and is capable of rescuing lifespan in *daf-2;daf-16* compound mutants but does not extend lifespan in wild-type animals (Lin et al., 2001). Mutations in the conserved gene *eak-7* promote activity of DAF-16 without any apparent alteration in its nuclear localization and cause a DAF-16 dependent increase

in longevity (Alam et al., 2010). Transgenic EAK-7::GFP constructs are constitutively localized to the plasma membrane by an N-myristoylation motif, suggesting that they regulate activity of nuclear DAF-16/FoxO indirectly through downstream intermediates (Alam et al., 2010). Mechanisms that directly regulate activity of DAF-16/FoxO in the nucleus are not fully understood.

DAF-16/FoxO activity promotes longevity in multiple contexts

DAF-16/FoxO promotes longevity independently of reduced insulin signaling. Ablation of germline stem cells, via laser surgery or a temperature-sensitive mutation of *glp-1*/Notch, causes an increase in DAF-16/FoxO nuclear localization and a *daf-16*-dependent increase in longevity (Arantes-Oliveira et al., 2002; Hsin & Kenyon, 1999; Lin et al., 2001). Importantly, molecular requirements for increased nuclear localization of DAF-16 in germline-ablated animals are distinct from IIS. Nuclear localization of DAF-16, and longevity, in germline ablated *glp-1* mutants requires a conserved ankyrin-repeat containing protein KRI-1, and TCER-1, a transcription elongation regulator, as well as nuclear hormone receptor DAF-12, and DAF-9, a cytochrome P450 that is necessary for production of DAF-12 ligands (Berman & Kenyon, 2006; Gerisch et al., 2001; Ghazi et al., 2009). KRI-1, DAF-9, and DAF-12 are dispensable for nuclear localization of DAF-16 in insulin signaling mutants (Berman & Kenyon, 2006). RNAi targeting *kri-1* does not reduce lifespan in *daf-2* mutants, suggesting that it is not required for increased longevity in the context of reduced insulin signaling (Berman & Kenyon, 2006). Genetic analysis suggests that *daf-12* and dafachronic acid biosynthesis genes play complex modulatory

roles in the lifespan of *daf-2*/IGFr mutants that may be independent of DAF-16 nuclear localization (Dumas et al., 2013; Dumas et al., 2010; Ghazi et al., 2009).

DAF-16/FoxO also plays roles in longevity mediated by changes in diet. Mutants that are defective in chemosensation due to cilia defects in sensory neurons, and ablations of individual gustatory and olfactory neurons extend lifespan through mechanisms that depend on DAF-16/FoxO. Extension of lifespan in sensory mutants can be due to reductions in the perception of food signals, or changes in perception of food-independent environmental factors (Alcedo & Kenyon, 2004; Apfeld & Kenyon, 1998). A dietary restriction regimen in which worms on solid plates are given low concentrations of bacterial food and transferred repeatedly as they exhaust the available food on the plate extends lifespan through a *daf-16*-dependent mechanism; DAF-16/FoxO activation in this context was proposed to depend on the C. elegans AMPKα2 subunit homologue AAK-2 rather than reduced IIS (Greer et al., 2007). Importantly, other dietary restriction protocols have been described to extend lifespan independently of DAF-16/FoxO and dependently on other transcription factors including *pha-4*/FoxA and *skn-1*/Nrf-2 (Bishop & Guarente, 2007; Houthoofd et al., 2003; Kaeberlein et al., 2006; Panowski et al., 2007). Taken together these data suggest that DAF-16/FoxO plays important, albeit context dependent, roles in mediating the impact of food perception and calorie restriction on lifespan.

The timing and location of DAF-16/FoxO action in lifespan control

To understand how reduced insulin signaling and DAF-16/FoxO regulate lifespan it is important to understand where they act to control longevity. Interestingly, evidence

suggests that lifespan regulation by reduced insulin signaling and DAF-16/FoxO activity may occur in distinct tissues. The long lifespan of *daf-2*/IGFr mutant animals can be reduced by transgenic expression of DAF-2/IGFr under heterologous promoters. Expression of DAF-2 under a ubiquitous promoter (*dpy-30p*) in *daf-2* mutants reduces the lifespan to nearly that of wild-type animals. A similar reduction is observed when DAF-2 is expressed under a neuronal promoter (*unc-14p*) while a much smaller reduction in lifespan is caused by intestinal DAF-2 expression (*ges-1p*) and no reduction in lifespan is caused by expression under a muscle specific promoter (*unc-54p*) (Wolkow et al., 2000). This result was supported by a parallel study that observed animals with mosaic expression of wild-type *daf-2* within a free chromosomal duplication that can be lost mitotically, in *daf-2* mutant backgrounds (Apfeld & Kenyon, 1998).

In contrast to studies rescuing *daf-2*/IGFr mutant lifepans, a separate study found that when the lifespan of *daf-2;daf-16* mutants is rescued by transgenic expression of DAF-16, the lifespan extension conferred by intestinal (*ges-1p*) expression is much larger than the lifespan extension conferred by neuronal expression (*unc-119p*) (Libina et al., 2003). The observation that neither neuronal nor intestinal expression of DAF-16 conferred as large an effect on lifespan as expression under its endogenous promoter suggests that DAF-16 activity in multiple tissues may be required for full lifespan extension (Libina et al., 2003). Importantly, a separate study found that intestinal expression of DAF-16/FoxO was sufficient to rescue the dauer diapause phenotypes of *daf-16* mutants, while the Libina study found that neuronal expression of DAF-16 was critical for dauer phenotypes, suggesting that further studies, perhaps using tissue specific knock-outs, will be necessary to resolved differences between heterologous expression studies that may be

caused by imperfect understanding of promoter specific expression patterns (Hung et al., 2014). While the use of different neuronal promoters is a caveat for direct comparison of the study by Libina and colleagues to the above study of heterologous DAF-2 expression, the results are nonetheless consistent with a model where reductions in neuronal insulin signaling control lifespan via non-cell-autonomous regulation of DAF-16/FoxO.

It is also critical to understand when IIS and DAF-16/FoxO activity act to promote lifespan. An RNA interference study was used to identify timepoints at which knockdown of DAF-2/IGFr activity was sufficient to extend lifespan, and timepoints at which knockdown of DAF-16/FoxO activity could reduce lifespan in *daf-2*/IGFr mutants. Results showed that knockdown of *daf-2/*IGFr starting at any time from first larval stage to day 4 of adulthood could extend lifespan, while initiation of *daf-2* RNAi at day 5 or later had little to no impact on lifespan (Dillin et al., 2002). In contrast, *daf-16*/FoxO RNAi starting as late as day 15 of adulthood caused large reductions in lifespan in *daf-2*/IGFr mutants (Dillin et al., 2002). This study suggests a mechanism through which reductions of insulin signaling in young adults produce high levels of DAF-16/FoxO activity throughout life, with diminishing DAF-16/FoxO activation when insulin signaling is reduced later in life. The molecular basis for such a mechanism remains unknown. The observations that RNAi is less efficient in neurons (the primary site of DAF-2 action) than in intestine (a critical site of DAF-16 action) and that declines in feeding in aged animals might reduce the efficiency of RNAi, are important caveats for interpretation of this study (Huang et al., 2004; Libina et al., 2003; Timmons & Fire, 2001; Wolkow et al., 2000).

Genetic studies of *C. elegans* have established an important role for the FoxO transcription factor DAF-16 in promoting increased lifespan. While insulin signaling is the most well characterized mechanism of DAF-16/FoxO regulation, other upstream regulatory processes including germline ablation and dietary restriction regulate DAF-16 activity. Evidence suggests that insulin signaling controls DAF-16 activity in part through cell non-autonomous mechanisms, with IIS activity in neurons in young adulthood establishing DAF-16 activity in multiple tissues during aging. Overall, an extensive body of evidence demonstrates that both reduced IIS and increased DAF-16/FoxO activity can dramatically delay onset of age-related mortality and decline, while extending median and maximum longevity in *C. elegans*.

Insulin signaling and FoxO family transcription factors are conserved regulators of longevity

One reason for performing initial genetic studies in *C. elegans* is its relative simplicity. Having identified insulin-like signaling as a regulator of longevity in *C. elegans*, it was reasonable to ask whether the role of insulin signaling and FoxO activity in aging and age-related pathology is conserved in more complex organisms.

The role of reduced insulin signaling and increased DAF-16/FoxO activity in longevity appears to be conserved from nematodes to the fruit fly *Drosophila melanogaster*. Mutation of the *Drosophila* insulin receptor substrate homologue *chico* extends lifespan through a mechanism that requires the sole *Drosophila* FoxO family member *dFoxo* (Clancy et al., 2001; Yamamoto & Tatar, 2011). Overexpression of *dFoxo* in the *Drosophila* fat body also extends lifespan, suggesting that FoxO activity is sufficient to

increase longevity in flies (Hwangbo et al., 2004). Given the large phylogenetic distance between worms and flies, these data suggest that the mechanisms underlying lifespan extension by increased FoxO activity may be highly conserved.

There is evidence that reduced activity of the insulin signaling pathway regulates longevity and age-related morbidities in mammals. Mice in which the insulin receptor is knocked out specifically in adipose tissue (Fat-specific-Insulin-Receptor-Knock-Out or FIRKO mice) are long lived and resistant to obesity (Blüher et al., 2003). Mice with mutations that block growth hormone signaling by preventing development of growth hormone producing cells in the pituitary, or knocking out the growth hormone receptor, are long lived and have reduced hepatic expression of IGF-1, reduced circulating IGF-1 levels, reduced insulin-levels, improved insulin sensitivity, delayed onset an reduced incidence of fatal neoplasia, delayed immune aging, and improved maintenance of cognitive functions in old animals, suggesting that reduced insulin and IGF-1 signaling may contribute to protection from multiple age related morbidities (Bartke, 2011; Flurkey et al., 2001; Freude et al., 2009; Ikeno et al., 2003; Ikeno et al., 2009; Killick et al., 2009; Kinney et al., 2001a; Kinney et al., 2001b; Masternak et al., 2009; Sharp & Bartke, 2005; Vergara et al., 2004). Knockout of pregnancy-associated-plasma-protein-A (PAPP-A), which can function to increase local IGF-1 availability by cleaving IGF-1 binding proteins, causes increased lifespan and delayed occurrence of fatal neoplasia in mice, further suggesting that reduced IGF signaling can increase lifespan in mammals (Conover et al., 2010).

The mammalian genome encodes four FoxO transcription factors with overlapping expression patterns and critical roles in development, making it difficult to determine the contributions of mammalian FoxO transcription factors to longevity. Phenotypes of FoxO knockout mice suggest that FoxO transcription factors may play roles in age-related pathologies including insulin resistance, osteoporosis and neoplasia (Greer & Brunet, 2005; Hesp et al., 2015; Paik et al., 2007). A recent study found that the lifespans of Foxo3 knockout mice are not extended by dietary restriction, suggesting that Foxo3 activity promotes longevity in dietary restricted mice (Shimokawa et al., 2015). Single nucleotide variants in the human insulin-like-growth-factor I receptor and FOXO3A loci are associated with longevity and multiple measures of improved health and delayed morbidity in centenarians, suggesting that IIS and FoxO activity may be correlated longevity in humans (Flachsbart et al., 2009; Joshi et al., 2017; Li et al., 2009; Suh et al., 2008; Willcox et al., 2008).

Taken together these data suggest that the effects of reduced insulin signaling on longevity are highly conserved. An improved understanding of the molecular mechanisms underlying the longevity phenotypes of IIS mutants in invertebrates may provide mechanistic insights into the basis of extended longevity and delayed morbidity that will be relevant to mammals.

Phenotypes of long lived insulin signaling mutants

A first step in finding out why reduced insulin signaling and DAF-16/FoxO activation extend lifespan is to study observable phenotypes of IIS mutants. Perhaps the most basic question about IIS and other long-lived mutants, is why changes that improve overall

survival are not selected for in natural populations. Evolutionary theory suggests that mutations that extend lifespan are likely to carry pleiotropies that prevent successful competition with wild-type animals (Williams, 1957). While *daf-2* mutants appear grossly normal, when they are placed in competition with wild-type animals under conditions of cycling food availability, they are rapidly lost from the population, suggesting that wild-type activity of the insulin receptor is necessary for worms to maximize reproduction and survival under conditions of variable nutrition likely found in nature (Jenkins et al., 2004).

Mutations in *daf-2* also confer resistance to a variety of environmental stresses, including high temperature, treatment with heavy metals, and infection by pathogenic bacteria (Barsyte et al., 2001; Garsin et al., 2003; Lithgow et al., 1995). Resistance to multiple types of cellular stress is a common feature of increased lifespan; primary cells from long-lived species are generally more tolerant to a variety of cellular stresses than those from related short-lived species, and mutations that confer increased longevity in mice also frequently cause increases in cellular resistance to various environmental stresses (Harper et al., 2007; Salmon et al., 2005). This suggests that resistance to molecular damage caused either by short-term stresses or long-term damage accumulation during aging may be a mechanism of lifespan extension. However, it is difficult to determine which stress resistance mechanisms are important for lifespan phenotypes. RNAi against a putative DAF-16 co-factor, *smk-1*, blocks lifespan extension in *daf-2* mutants, while reducing their resistance to oxidative stress (paraquat) but not heat (Wolff et al., 2006). Similarly, a loss of function mutation in the transcription factor *skn-1* suppresses an oxidative stress resistance phenotype (arsenite treatment) of strong *daf-2* loss-of-function

mutants without suppressing their increased longevity phenotype when animals are cultured at 20°C (Ewald et al., 2015). The separability of specific-stress resistance phenotypes from longevity phenotypes will make it challenging to make inferences about molecular mechanisms of longevity phenotypes based solely upon mechanisms of stress resistance.

In addition to their stress resistance phenotypes *daf-2*/IGFr mutants have substantial changes in energy metabolism relative to wild-type animals. Mutations in *daf-2*/IGFr cause the intestines of living worms to appear visibly darker under a dissecting microscope; this corresponds to increased fat storage, measured both by staining with lipophilic dyes and mass spectroscopy (O'Rourke et al., 2009). Increased fat accumulation in *daf-2*/IGFr mutants may be related to their developmental phenotypes. At high temperatures, *daf-2*/IGFr mutants arrest in the dauer diapause, and even at permissive temperatures they pass through the pre-dauer L2D larval phase which drives increased fat accumulation; this has been proposed to be responsible for increased fat storage in *daf-2*/IGFr mutant adults (Golden & Riddle, 1984; Wolkow et al., 2000). In support of this model, treating animals with RNAi targeting *daf-2*/IGFr at adulthood was reported to reduce fat content, consistent with a role for reduced IIS in mobilizing stored nutrients during dietary restriction (Wang et al., 2008).

In summary, reductions in insulin signaling cause several interesting physiological phenotypes in addition to increased lifespan. These include resistance to a variety of environmental stresses, and changes in fat metabolism. However, many of these phenotypes are somewhat context dependent, with different IIS reducing interventions

having opposite effects on fat content and resistance to some environmental stressors being genetically separable from longevity. Based on the information available it will likely be difficult to reach a mechanistic understanding of lifespan extension using only hypotheses based on non-longevity phenotypes of long-lived animals.

Identification of downstream transcriptional targets of DAF-16/FoxO that contribute to longevity

DAF-16/FoxO is a transcription factor, and DAF-16/FoxO-dependent increases in lifespan are strongly associated with localization of DAF-16/FoxO to the nucleus, and with increased transcriptional activity of nuclear DAF-16/FoxO. Thiss suggests that DAF-16/FoxO activity increases longevity by influencing transcription of downstream target genes, though this has not been thoroughly testes using *daf-16*/FoxO mutants that are specifically defective in DNA binding (Alam et al., 2010; Lin et al., 2001; Murphy et al., 2003). Identification of transcriptional targets of DAF-16/FoxO followed by functional testing for effects on longevity can provide insights into the downstream mechanisms through which increased DAF-16/FoxO activity increases longevity. A number of studies have attempted to identify transcriptional targets of DAF-16/FoxO that may contribute to longevity using unbiased approaches (Amrit et al., 2016; Chen et al., 2015; Lee et al., 2003; McCormick et al., 2012; McElwee et al., 2003; McElwee et al., 2004; Murphy, 2006; Murphy et al., 2003; Oh et al., 2006; Schuster et al., 2010).

To determine whether changes in transcription observed in profiling studies represent a DAF-16-dependent transcriptional program, it is critical to identify sites in the genome at which DAF-16 is likely to bind, and to validate at least a few direct DAF-16 target genes

that can serve as positive controls. *In vitro* studies of DAF-16/FoxO binding to random oligonucleotides have determined that both DAF-16 and some vertebrate FoxO transcription factors bind robustly to a conserved TTGTTTAC motif, referred to as a DAF-16 binding element or DBE (Furuyama et al., 2000). However, this motif occurs at least once within 5kb upstream of 78% of annotated genes in the worm genome, suggesting that the presence of DBEs alone is a poor method for identifying DAF-16 targets (Kenyon & Murphy, 2006).

Published studies have used chromatin immunoprecipitation and DamID to identify genomic sites at which DAF-16 binds (Oh et al., 2006; Schuster et al., 2010). The study using DamID was able to correlate genomic sites of DAF-16 binding with existing transcriptional profiling data to generate a high priority list of 65 genes whose transcription is upregulated by direct binding of DAF-16/FoxO to their promoters (Schuster et al., 2010). This set is enriched for genes that are transcriptionally upregulated in a DAF-16 dependent manner in *daf-2* mutants, suggesting that DAF-16 principally acts directly to increase transcription of its target genes (Schuster et al., 2010). A handful of DAF-16 target genes including the superoxide dismutase *sod-3*, and the metallothionein family member *mtl-1* have been validated as transcriptionally upregulated DAF-16 targets in multiple studies (Murphy, 2006; Murphy et al., 2003). Importantly, there are statistically significant overlaps between genes that are transcriptionally regulated or bound by DAF-16 and orthologous genes that are bound or transcriptionally regulated by *Drosophila* dFOXO, suggesting that some transcriptional outputs of DAF-16/FoxO activity, such as its effects on lifespan, are broadly conserved (Alic et al., 2011).

The field of identifying DAF-16/FoxO transcriptional targets that are involved in longevity has been defined by a seminal study performed by Cynthia Kenyon and colleagues (Murphy et al., 2003). This study used microarrays to compare transcriptomes of animals carrying multiple alleles of *daf-2*/IGFr and *age-1*/PI3K to wild type, as well as comparing a single *daf-2* mutant to compound *daf-2;daf-16(0)* mutants. Importantly, the study controlled for genetic background by also comparing isogenic animals treated with *daf-2* RNAi or *daf-2* and *daf-16* RNAi. They also compared multiple time points to control for systematic errors introduced by longitudinal changes in the early adult transcriptome.

The Kenyon study identified two classes of target genes: genes that were consistently upregulated in a DAF-16/FoxO-dependent manner in *daf-2* mutants were termed Class I DAF-16 targets and genes that were consistently downregulated in a DAF-16/FoxO-dependent manner in *daf-2* mutants were termed Class II DAF-16 targets. By identifying putative DAF-16/FoxO targets that changed robustly over many arrays they were able to prioritize a relatively small subset of 58 genes to test for lifespan effects using RNAi. RNAi of Class I DAF-16 targets was tested for the expected suppression of extended life span in *daf-2*/IGFr mutants, while RNAi of genes that were transcriptionally downregulated in long-lived strains in a DAF-16-dependent manner was tested for lifespan extension in wild type animals. Strikingly, fifty out of fifty-eight genes that were tested affected lifespan, with 49 doing so in the expected direction. Most effects were small in magnitude (under twenty percent reductions in lifespan compared to *daf-2* mutants on vector RNAi for DAF-16 upregulated genes); however, RNAi against one target, *dod-1*, reduced lifespan of *daf-2* mutants by 30% to 40% in two trials. Biological

functions of DAF-16 targets affecting lifespan will be discussed below. The magnitude of the effects observed led the authors to speculate that DAF-16 activity extends lifespan through additive effects of many target genes. It is worth noting that the high percentage of tested DAF-16 targets with effects on lifespan observed in this report is not typical of subsequent efforts to test candidate DAF-16 targets lifespan effects; this may suggest that this study was particularly efficient at enriching for DAF-16 targets with roles in lifespan, or that the experimental conditions used were highly sensitized for lifespan effects, or some combination of the above (Chen et al., 2015; Murphy, 2006). The study was performed in a sterile (*fer-15;fem-1*) background to avoid the use of pharmacological agents (5-fluoro-2'-deoxyuridine, FUDR, which inhibits DNA synthesis) to inhibit worm fertility, and the controls had shorter lifespans than are usually observed in wild-type worms which could have sensitized these animals to lifespan effects (Murphy et al., 2003).

Unexpectedly, the Kenyon study identified two putative transcription factor binding sites that were enriched upstream of genes with DAF-16-dependent changes in transcriptional changes using an unbiased algorithmic approach; in addition to the expected DBE being enriched in the promoters of both Class I and Class II targets, they identified another motif (CTTATCA) that was enriched in the upstream regulatory region of both Class I and Class II targets, in various combinations with the DBE. They named this putative transcription factor binding site the DAF-16 associated element or DAE. A recent study was able to identify this motif as the binding site for another transcription factor, PQM-1 (Tepper et al., 2013). A PQM-1::GFP transgene is exported from the nucleus under reduced IIS, and its nuclear localization is anti-correlated with nuclear localization of

DAF-16, suggesting that PQM-1 may predominantly act to promote the transcription of Class II genes which are downregulated when PQM-1 is absent during reduced IIS (Tepper et al., 2013). Importantly, the ChIP-seq data sets used to identify PQM-1 show that the promoters of both Class I and Class II targets are highly enriched for PQM-1 binding, suggesting that the PQM-1 may influence lifespan, at least in part, altering transcription of both Class I and Class II targets (Tepper et al., 2013).

Several other studies have attempted to identify transcriptional targets of DAF-16 that are involved in longevity (Amrit et al., 2016; Chen et al., 2015; Halaschek-Wiener et al., 2005; Lee et al., 2003; McCormick et al., 2012; McElwee et al., 2003; McElwee et al., 2004). Most of these studies have involved comparisons of *daf-2*/IGFr mutant animals to *daf-2;daf-16*/FoxO compound mutants (Chen et al., 2015; Halaschek-Wiener et al., 2005; McElwee et al., 2003; McElwee et al., 2004). Germline ablated animals have also been profiled (Amrit et al., 2016; McCormick et al., 2012), and one study has bioinformatically identified targets based on the presence of DBE's in the promoter of *C. elegans* and orthologous *Drosophila* genes (Lee et al., 2003). Many of these studies did not perform robust filtering to remove false positives, all have reported testing fewer targets than the initial profiling study and have found a lower proportion of targets with lifespan in effects in the direction that would be expected from the change in transcription (Murphy, 2006).

A major issue in identifying a consensus set of genes that are transcriptionally regulated by DAF-16 has been low agreement among published microarray studies. For example, out of 3032 putative DAF-16 transcriptional targets identified by the first three published

profiling studies, only ninety were identified in all three (McElwee et al., 2003; McElwee et al., 2004; Murphy, 2006; Murphy et al., 2003). Differences between datasets generated by different groups may vary due to lab-to-lab changes in how worms were cultured and RNA was collected. Subtle differences in genetic background, developmental stage, and synchronization could also affect the DAF-16 transcriptome (Kenyon & Murphy, 2006). Despite differences between studies, many "gold standard" DAF-16 targets have been identified in multiple studies. These include genes that have been identified both based on binding of DAF-16 to their promoters and DAF-16 dependent transcriptional regulation. A meta-analysis has used an unbiased voting algorithm to identify a rank-ordered list of DAF-16 targets that are consistently differentially regulated in various micro-array studies (Murphy, 2006; Schuster et al., 2010; Tepper et al., 2013).

Most studies that have functionally tested candidate DAF-16 target genes for effects on lifespan have done so by inactivating them using feeding RNAi starting at young adulthood. While RNAi permits high throughput studies on identical genetic backgrounds, the approach has major caveats. RNAi can cause off-target knockdowns of multiple genes or can fail to fully inactivate the target gene (Qiu et al., 2005). Some tissues, including neurons, which are critical regulators of lifespan, are refractory to RNAi (Timmons & Fire, 2001). In addition, while performing RNAi at adulthood rather than using mutants prevents developmental defects from confounding lifespan studies, it may cause false negatives when testing genes that are principally expressed prior to adulthood but do affect lifespan. Whole genome sequencing of mutagenized strains has made loss of function mutations in many genes publicly available, and CRISPR/Cas9-

based gene editing now permits straightforward creation of targeted knockouts (Friedland et al., 2013; Thompson et al., 2013). In the future it will be important to perform functional studies of DAF-16/FoxO targets that regulate lifespan using mutants in addition to RNAi.

To perform detailed genetic studies of DAF-16 targets in longevity, it will be necessary to prioritize DAF-16/FoxO targets that are likely to be involved in lifespan determination. A method of prioritizing DAF-16 targets has been revealed by recent studies demonstrating that transcriptional isoforms of DAF-16 have distinct roles in lifespan determination (Chen et al., 2015; Kwon et al., 2010; Lee et al., 2001). We conducted an analysis of isoform-specific DAF-16 mutants which demonstrated that DAF-16A and DAF-16F isoforms act hierarchically to promote lifespan. Mutations that specifically eliminate DAF-16A partially suppress the long lifespans of *daf-2*/IGFr mutants, while mutations that specifically eliminate DAF-16F do not reduce lifespan, suggesting that function of the DAF-16F isoform only becomes critical for *daf-2*/IGFr mutant longevity when *daf-16a* is absent (Chen et al., 2015).

The transcriptomes of *daf-2*/IGFr mutants were then compared to *daf-2;daf-16* compound mutants harboring isoform-specific *daf-16* mutations, to identify target genes whose transcription was influenced strongly by *daf-16a* or equally influenced by DAF-16A and DAF-16F. Importantly, this analysis verified multiple "gold-standard" DAF-16 target genes that have identified by multiple previous profiling studies, and several DAF-16 target genes that have been previously validated to have lifespan effects. A set of twenty DAF-16A specific, nine DAF-16A/F redundant, and two DAF-16F targets was tested for

lifespan effects, using genetic mutants rather than RNAi. RNAi of two genes, a single DAF-16 upregulated Class I target (*gst-20*) and a single DAF-16 downregulated Class II target (*srr-4*), affected lifespan in the expected direction. Interestingly, both effects were observed when worms were cultured on the bacterial strain HT115 (used in RNAi experiments) but not on the bacterial strain OP50 (the standard for non-RNAi studies), highlighting a potential caveat of translating lifespan effects from RNAi-based studies to studies using mutants (Chen et al., 2015).

Due to the substantial, and potentially conserved, effects of DAF-16 activity on lifespan, delineating the transcriptional program that promotes longevity downstream of DAF-16 activation has been a subject of intense interest. At this point a large body of studies have used genome wide transcriptomic approaches to identify genes that contribute to lifespan extension downstream of DAF-16. Identifying a consensus set of DAF-16 target genes has been difficult, due, in part, to the sheer number of DAF-16 dependent transcriptional changes that can be identified. Nonetheless, multiple approaches including direct analysis of DAF-16 binding sites in the genome, genetic studies of *daf-16* isoforms, individual studies that robustly enrich for DAF-16-dependent regulatory events over multiple transcriptomic experiments, transcriptomic profiling of specific tissues, and meta-analyses of publicly available data have identified many consensus DAF-16/FoxO transcriptional targets, some of which do affect lifespan (Chen et al., 2015; Kaletsky et al., 2016; Murphy et al., 2003; Schuster et al., 2010; Tepper et al., 2013). At this point it is reasonable to ask what biological processes have been found to contribute to increased longevity.

Downstream mechanisms of longevity regulation by DAF-16/FoxO activity

Multiple studies have identified transcriptional targets of DAF-16 that contribute to the long lifespans of *daf-2*/IGFr mutants. An increasing number of functional studies are now moving beyond identifying individual target genes that are critical for longevity to characterizing the biological processes they are involved in. This work may provide insight into conserved cellular functions that can be targeted to extend lifespan and delay or prevent age-related morbidity. Here we will classify biological processes that are regulated by DAF-16 that are likely to contribute to longevity in *C. elegans*, and that may be conserved.

DAF-16/FoxO regulates signaling genes

DAF-16 activation alters expression of several genes that are recognizable components of conserved signaling pathways, including genes that alter its own activity. DAF-16 activity robustly increases transcription of AKT-2, which acts in IIS to inhibit DAF-16 activity (Chen et al., 2015; Paradis & Ruvkun, 1998). *akt-2* mutants are long-lived, suggesting that transcriptional upregulation of AKT-2 by DAF-16 acts in a negative feedback loop to increase the sensitivity of DAF-16 to downregulation by IIS and limit longevity (Alam et al., 2010).

In contrast, DAF-16 also activates transcription of *aakg-4*, an atypical AMP-kinase γ-subunit that has been shown to act in a positive feedback loop to increase DAF-16 activity (Chen et al., 2015; Chen et al., 2013; Tullet et al., 2014). A predicted *aagk-4* null mutation completely abrogated lifespan extension in a *daf-2*/IGFr mutant background and

also caused reduced upregulation of other canonical DAF-16 target genes in a *daf-2*/IGFr mutant background, suggesting that it influences lifespan by positively regulating DAF-16 activity (Tullet et al., 2014).

scl-1 is another gene that is transcriptionally upregulated by DAF-16/FoxO and that may function in both lifespan and signaling. The *scl-1* locus encodes a predicted secreted protein with homology to the mammalian cysteine rich secreted protein family (Ookuma et al., 2003). Though it was originally identified in a targeted qPCR study rather than a transcriptomic approach, the *scl-1* transcript is reproducibly upregulated in IIS mutants (Hsu et al., 2003; Ookuma et al., 2003; Tullet et al., 2014). Strikingly, *scl-1* RNAi was reported to completely abrogate the lifespan extension observed in long-lived *daf-2(e1370)* mutants (Ookuma et al., 2003). The function of *scl-1* remains obscure; however, its annotation as a secreted protein suggests that it could function in cell non-autonomous signaling (Ookuma et al., 2003).

Increased DAF-16/FoxO activity also controls longevity by downregulating signaling molecules. The insulin-like peptide INS-7 is transcriptionally downregulated in *daf-2*/IGFr mutants in a DAF-16-dependent manner, and *ins-7* RNAi increases lifespan (Chen et al., 2015; Murphy et al., 2003). An INS-7::GFP transgene under the control of its own promoter is expressed in the intestine and neurons, and constitutive expression of INS-7 in the intestine prevents constitutive intestinal DAF-16/FoxO expression from upregulating transcriptional activity of DAF-16/FoxO in other tissues, suggesting that disinhibition of INS-7 expression permits FoxO-to-FoxO signaling from intestine to other tissues (Murphy et al., 2007).

DAF-16 activity has also been found to downregulate activity of *daf-15*/Raptor (Jia et al., 2004). *daf-15*/Raptor mutants have reduced TOR activity and are long lived, suggesting that downregulation of *daf-15*/Raptor by DAF-16/FoxO may promote increased longevity through the TOR pathway.

In sum, longevity regulation by DAF-16 depends in part on differential expression of genes in multiple signaling pathways, each of which likely has downstream effects on transcription. This is consistent with the observation that many genes that are transcriptionally regulated by DAF-16 are not observed to have DAF-16 bound to their promoters (Schuster et al., 2010).

DAF-16/FoxO regulates other transcriptional regulators

In addition to upstream signaling genes, many DAF-16 targets with effects on lifespan are themselves direct regulators of gene expression. SKN-1/Nrf-2 has been reported to be transcriptionally upregulated in a DAF-16/FoxO-dependent manner in germline ablated *glp-1* mutants, and *skn-1* mutation fully abrogates lifespan extension in germline ablated *glp-1* mutants, suggesting that DAF-16 may extend lifespan in part through indirect activation of SKN-1 targets in germline ablated animals (McCormick et al., 2012; Steinbaugh et al., 2015).

Another transcription factor, *mdl-1*, is homologous to the mammalian Mad family of transcription factors and is both a target of DAF-16 direct binding to its promoter, and DAF-16 dependent transcriptional upregulation in *daf-2*/IGFr mutants (Chen et al., 2015; Riesen et al., 2014; Schuster et al., 2010). A predicted null mutation in *mdl-1* partially suppresses the lifespan extension phenotype of a *daf-2*/IGFr mutant, suggesting that

transcriptional upregulation of MDL-1/Mad by DAF-16/FoxO promotes increased lifespan through activation of MDL-1 target genes (Riesen et al., 2014).

Germline-ablated *glp-1* mutant animals also transcriptionally upregulate a transcription elongation factor, TCER-1, and an RNA binding protein, PHI-62, in a manner that depends on both DAF-16/FoxO and DAF-12/NHR (Ghazi et al., 2009; McCormick et al., 2012). RNAi targeting either *phi-62* or *tcer-1* blocks the long lifespan of germline-ablated mutants and downregulates transcription of some other DAF-16 target genes, suggesting that TCER-1 and PHI-62 promote increased lifespan by promoting transcriptional regulation of other DAF-16 targets (Amrit et al., 2016; Ghazi et al., 2009; McCormick et al., 2012). Interestingly, both of these genes are dispensable for lifespan in the context of *daf-2*/IGFr mutants, suggesting that DAF-16 can initiate the transcriptional events that are required for increased longevity independently of these co-regulators when it is activated by reduced IIS (Amrit et al., 2016; Ghazi et al., 2009; McCormick et al., 2012).

The DAF-16-dependent upregulation of both signaling pathway genes and other transcription regulators may help explain the large number of genes that are differentially regulated in a DAF-16/FoxO dependent manner, as well as the observation that many genes that are robustly transcriptionally regulated by DAF-16 do not appear to be direct targets of DAF-16 binding. In general, this evidence may suggest that DAF-16/FoxO acts as a master regulator of transcription, controlling the activation of signaling pathways and transcriptional programs in response to environmental stresses, and promoting increased longevity through indirect activation of downstream responses.

DAF-16/FoxO activity alters fat metabolism

While the observation that DAF-16 may influence longevity through activation of downstream signaling and transcription related proteins is interesting from the perspective of understanding the regulatory logic of pro-longevity responses, it is also somewhat disappointing for those of us who hope that understanding an enzymatic program that is sufficient to increase lifespan and delay multiple aspects of age-related functional decline could provide insights into the molecular causes of aging. Interestingly, an emerging body of work has begun to identify DAF-16 target genes that may contribute more directly to longevity, improving to our understanding of biological processes that may delay aging.

DAF-16/FoxO activity extends longevity in part by upregulating members of the lipase family, encoded by the genes *lipl-1-4*. *lipl-4* was first identified in an RNAi screen for genes that increase fat accumulation in germline-ablated animals; however, subsequent transcriptomic profiling studies have confirmed that it is regulated in a DAF-16-dependent manner in germline-ablated and *daf-2*/IGFr mutant strains (Chen et al., 2015; Wang et al., 2008). RNAi targeting *lipl-4* reduces lifespan extension in both *daf-2*/IGFr mutants and germline-ablated *glp-1* mutants, suggesting that LIPL-4 activity promotes longevity (Wang et al., 2008). Strikingly, overexpression of LIPL-4 in the intestine increases lifespan of wild-type worms, demonstrating that LIPL-4 activity is sufficient to increase lifespan (Wang et al., 2008). Excitingly, overexpression of LIPL-4 has been shown to drive increased expression of polyunsaturated fatty acid species that are sufficient to induce autophagy in both worms and mammalian cells (O'Rourke et al.,

2013). Furthermore, components of the autophagy machinery are necessary for lifespan extension in LIPL-4 overexpressing animals, suggesting that LIPL-4 activity extends lifespan by directly activating turnover of damaged proteins and cellular organelles via inducing macro-autophagy (Lapierre et al., 2011). However, the longevity extension caused by LIPL-4 has also been reported to require a signaling mechanism involving a lipid chaperone, LBP-8, translocating to the nucleus and activating nuclear hormone receptors (NHR-49 and NHR-80) suggesting that a downstream transcriptional response is also involved in LIPL-4 mediated lifespan extension (Folick et al., 2015).

Interestingly, other members of the lipase family are also involved in longevity regulation. DAF-16/FoxO activity has been reported to upregulate *lipl-1*, *lipl-2*, and *lipl-3* in addition to *lipl-4* in *daf-2*/IGFr mutants (Chen et al., 2015). LIPL-1 and LIPL-3 have been reported to be transcriptionally induced upon fasting, and overexpression of LIPL-1 or LIPL-3 modestly extends lifespan in wild-type animals (O'Rourke & Ruvkun, 2013). A predicted null mutant in *lipl-2* partially suppressed lifespan in *daf-2*/IGFr mutants, and *lipl-2* RNAi reduced longevity in germline-ablated *glp-1* animals (Ghazi et al., 2009) (unpublished data). A robust genetic analysis to determine the contribution of each lipase to the longevity of *daf-2*/IGFr or germline ablated mutants has not been reported.

A recent analysis of the transcriptome of germline-ablated animals revealed DAF-16/FoxO-dependent upregulation of both lipid anabolic and catabolic genes, including multiple genes encoding predicted diacyl-glycerol-acyl-transferase (DGAT) enzymes, which are involved in synthesizing triglycerides from free fatty acids (Amrit et al., 2016). Strikingly both mutation and RNAi against two DGAT-encoding genes, *dgat-2* and *acs-*

22, suppressed lifespan extension in germline ablated *glp-1* animals (Amrit et al., 2016). The lifespan effects of the mutations were dependent on bacterial food source; suppressing longevity when worms were cultured on *E.coli* strain HT115 but not on OP50 (Amrit et al., 2016). Food source differences in the effects of downstream lifespan regulators have now been reported in multiple studies (Amrit et al., 2016; Chen et al., 2015).

Several other putative fat metabolism genes have been reported to be regulated by DAF-16/FoxO, including fatty acid desaturases and lipid binding proteins (Murphy, 2006). RNAi against fatty acid desaturases was reported to reduce lifespan in *daf-2*/IGFr mutants (Murphy et al., 2003).

The initial insight that *daf-2*/IGFr and *glp-1*/Notch germline ablated animals have differences in fat storage relative to wild-type mammals has now been supported by a few mechanistic studies suggesting that fat metabolism enzymes contribute to increased longevity. Since the insulin and IGF pathways broadly regulate metabolism in mammals, these insights may ultimately reveal conserved mechanisms that increase longevity and delay age-related functional decline. Robust genetic and metabolic studies of multiple fat metabolism genes will be useful to identify specific lipid species that modulate longevity and identify the mechanism by which they affect downstream cellular processes such as autophagy.

DAF-16/FoxO activity promotes autophagy and mitophagy

Autophagy is induced in multiple contexts of increased lifespan, including *daf-2*/IGFr mutants, germline-ablated mutants, and dietary restriction (Gelino & Hansen, 2012; Hansen et al., 2008; Jia & Levine, 2007; Lapierre et al., 2011; Lapierre et al., 2015; Meléndez et al., 2003). RNAi-based knock-down of core components of the autophagy machinery abrogates lifespan extension in *daf-2*/IGFr mutants, and in many other long-lived contexts, suggesting that recycling of damaged proteins and organelles through autophagy may be a core mechanism of lifespan extension downstream of multiple pathways (Hansen et al., 2008; Lapierre et al., 2011; Meléndez et al., 2003).

DAF-16/FoxO-dependent upregulation of DCT-1, an orthologue of mammalian NIX/BNIP3L and BNIP3 mitochondrially expressed receptors that target mitochondria for autophagic degradation (termed mitophagy), has been observed in *daf-2*/IGFr mutants (Palikaras et al., 2015). RNAi against DCT-1 reduces lifespan in *daf-2*/IGFr mutants, as does RNAi against another core component of the mitophagy machinery, the PTEN induced mitochondrial kinase PNK-1 (Palikaras et al., 2015). This data suggests that increased expression of DCT-1 and PNK-1, and subsequent increased turnover of damaged mitochondria, promote lifespan downstream of reduced insulin signaling and DAF-16/FoxO. Interestingly *dct-1* and *pnk-1* deletion mutants had reduced resistance to heat and oxidative stressors (paraquat) relative to wild-type siblings, suggesting that increased mitophagy may be responsible for some aspect of increased stress resistance in *daf-2*/IGFr mutants (Palikaras et al., 2015).

Reduced insulin signaling may regulate autophagy through multiple mechanisms. *daf-2*/IGFr mutants have been observed to induce autophagy independently of DAF-16/FoxO in some tissues. In addition, it remains unclear whether induction of autophagy by lipase activity interacts specifically with mitophagy to promote longevity. Nonetheless, alterations in autophagy which produce delays in age-related accumulation of molecular damage, may provide a mechanistic basis for lifespan extension by DAF-16/FoxO.

DAF-16/FoxO activity promotes proteostasis and stress response

Increased autophagy may contribute to proteostasis by eliminating damaged proteins. Other proteostasis mechanisms have also been shown to contribute to lifespan extension downstream of DAF-16/FoxO activation. HSF-1/Heat-shock-factor promotes proteostasis by upregulating expression of molecular chaperones called heat-shock-proteins, whose functions include binding to unfolded proteins to prevent them from aggregating. RNAi against HSF-1 fully abrogates lifespan extension in *daf-2*/IGFr mutants, and DAF-16/FoxO and HSF-1 act together to transcriptionally upregulate expression of several small heat shock proteins, including *hsp-16.1*, *hsp-16.49*, *hsp-12.6*, and *sip-1* (Hsu et al., 2003). RNAi against each of these small heat shock proteins partially reduced lifespan in *daf-2*/IGFr mutants, and heat shock protein overexpression is sufficient to extend lifespan, strongly suggesting that increased DAF-16/FoxO activity extends lifespan, in part, through promoting expression of chaperones that prevent protein aggregation (Hsu et al., 2003; Walker & Lithgow, 2003). Strikingly, RNAi against each of these heat shock proteins also accelerated the age-related aggregation of Huntington's-like polyglutamine-repeat containing proteins expressed in *C. elegans*

muscle, suggesting that heat-shock protein expression may both increase lifespan and protect against an age associated morbidity (Hsu et al., 2003).

Several other classes of genes that could protect against or repair damage to proteins are upregulated in a DAF-16/FoxO-dependent manner in long lived mutants. These include genes encoding glutathione-S-transferases, which can protect against oxidative damage, as well as genes such as metallothionein that may detoxify xenobiotics (Murphy, 2006). RNAi against *gst-4*, and a loss of function mutation in *gst-20* have been reported to slightly reduce lifespan in *daf-2*/IGFr mutants, suggesting that these genes contribute to longevity (Chen et al., 2015; Murphy et al., 2003). As these genes may act partially redundantly, it would be of interest to construct compound mutants eliminating multiple DAF-16/FoxO regulated glutathione-S-transferases to determine whether greater effects on longevity are observed than in single mutants.

Overall, a substantial body of evidence supports the hypothesis that DAF-16/FoxO activity may promote longevity by improving proteostasis and preventing protein aggregation. Studies of these mechanisms, including studies of whether aggregation of endogenous proteins limits lifespan in *C. elegans*, may provide opportunities to connect the mechanistic basis of lifespan extension in nematodes to important mammalian pathologies.

Conclusions and Future Directions: the end of the beginning of identifying downstream longevity genes

Over twenty years since the cloning of *daf-2*/IGFr, *age-1*/PI3K, and *daf-16*/FoxO revealed that an insulin-like signaling pathway extends lifespan and delays visible signs of aging in *C. elegans*, an extensive body of work has begun using this pathway as a key to understand the fundamental biological mechanisms regulating the aging process. Studies of the signaling pathways upstream of DAF-16/FoxO activity, and the signaling and transcription genes it regulates, have outlined a complex regulatory system in which various stresses such as dietary restriction and germline-stem-cell arrest activate DAF-16/FoxO. DAF-16 in turn activates downstream stress response regulators, such as *skn-1*/Nrf-2, that combine to produce short term stress responses and long-term increases in longevity.

Both unbiased transcriptome profiling and hypothesis-based experiments have begun to reveal the downstream molecular program that dramatically delays aging in *daf-2*/IGFr mutants. These studies can help us both reject and confirm hypotheses about the molecular mechanisms that delay mortality and functional aging. Genetic analysis of compound mutants has largely disproven the hypothesis that *daf-2*/IGFr mutations extend lifespan by upregulating superoxide dismutase genes to protect against free radical damage (Doonan et al., 2008). Conversely, the hypotheses that triglyceride catabolism, autophagy, autophagic degradation of damaged mitochondria, and chaperone-mediated protection from aggregation-prone misfolded proteins, play roles in longevity extension seem to, thus far, be consistent with the available evidence. (Although a recent study

suggests that the benefits of autophagy may be context-dependent (Wilhelm et al., 2017)).

While the existing body of work has begun to produce fascinating insights, much work remains to be done. There is substantial variation in the results when transcriptomes of similar long-lived and short-lived strains are profiled in different labs. From a practical perspective, profiling strategies that robustly eliminate false positives over multiple experiments have had the most success at identifying target genes with lifespan effects (Murphy, 2006). Availability of affordable RNA-seq now makes it reasonable to conduct many profiling experiments and facilitates direct comparison between data sets. DAF-16 targets should be profiled using multiple combinations of long-lived strains, in the same hands, to identify targets that are robust across long-lived strains, and targets that are context unique. These experiments should be conducted in multiple labs and compared directly to eliminate systemic artifacts. Ultimately, using this approach, longevity target gene profiling can be "finished"; it will be possible to look up the degree to which any transcript is changed in various long-lived context, and to perform meta-analyses to identify new classes of longevity targets.

There is also substantial variation between labs in methodologies for testing target genes for longevity effects. Most groups use RNAi as a first pass to knock down genes of interest, but candidate testing for reduced longevity is performed using several different *daf-2*/IGFR alleles. There is substantial variation in lifespan between *daf-2*/IGFr alleles, and it is unclear whether different long-lived contexts will be equally responsive to lifespan effects. One might imagine that longer-lived *daf-2*/IGFr mutants will be more

sensitive to measurable reductions in lifespan, simply due to the larger phenotypic difference from wild-type in which to see an effect, or that they will be less sensitive, for example due to higher expression of many partially redundant pro-longevity programs. This type of understanding can best be developed by testing longevity regulating genes in multiple long-lived backgrounds using null-mutations. Given that both large numbers of null mutations are available, and that gene-editing approaches are available to make targeted mutations, it makes sense to test longevity targets using null alleles, unless there is a reason to suspect that a developmental pleiotropy will necessitate the use of RNAi at adulthood.

In addition, many enzymes that may play roles in longevity, such as lipases, fatty acid desaturases, and glutathione-S-transferases, represent potentially functionally redundant gene classes. The best way to determine whether a class of enzymes may be involved in lifespan extension will be to make compound mutants harboring multiple null mutations in potentially redundant genes, and test for lifespan effects. Finally, new technologies, including gene-editing mediated recombineering to generate single copy insertions of genes under heterologous promoters, or with rationally designed gain-of-function mutations, will enable more robust identification of genes that are sufficient to increase lifespan (Paix et al., 2016). This program represents a great deal of effort, perhaps eventually made less tedious by the availability of automated systems for performing lifespans (Stroustrup et al., 2013). However, the prize may be a detailed understanding of the molecular mechanisms underlying lifespan extension, and in turn normal aging. This will ultimately be vital to develop rational interventions to increase the portion of human lifespan that we can enjoy in good health.

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Chapter 4 The *Caenorhabditis elegans* carboxylesterase family member CEST-1 promotes longevity

Abstract

Although the role of FoxO transcription factors in promoting longevity is well established, how they do so is poorly understood. In the nematode *Caenorhabditis elegans*, the FoxO transcription factor DAF-16 extends life span in the contexts of reduced insulin-like signaling (ILS) and germline ablation. To gain insight into mechanisms underlying DAF-16/FoxO-dependent longevity, we used whole transcriptome profiling to define a set of 46 *dal* (*D*AF-16/FoxO target *a*ssociated with *l*ongevity) genes that are concordantly regulated in animals with either reduced ILS or absent germlines. One such gene, *cest-1*, is induced in a DAF-16/FoxO-dependent manner in *daf-2* mutants with reduced ILS and *glp-1* mutants that lack a germline. *cest-1* was both necessary for full life span extension in the context of reduced ILS and sufficient to extend life span when overexpressed. *cest-1* encodes a protein with a predicted signal peptide, a central domain with homology to carboxylesterases, and a putative transmembrane domain near the carboxyterminus. A functional CEST-1::GFP fusion protein is expressed specifically in the intestine and localizes to the apical plasma membrane. We propose that CEST-1 influences life span by acting within the intestinal lumen to either degrade a substance that shortens life span or generate a metabolite that promotes longevity. As human carboxylesterases are hypothesized to play roles in xenobiotic and lipid metabolism, understanding how CEST-1 promotes longevity in

C. elegans may yield insights into potential roles of carboxylesterase family members in influencing aging in humans.

Introduction

FoxO transcription factors (TFs) have emerged as important regulators of the aging process. They extend life span across taxa and influence phenotypes associated with aging-related pathology in mouse models of human disease (Bartke, 2011; Blüher et al., 2003; Clancy et al., 2001; Joshi et al., 2017; Lin et al., 1997; Shimokawa et al., 2015; Yamamoto & Tatar, 2011). Common genetic variants at the human FOXO3 locus are associated with longevity and protection against aging-associated diseases, suggesting that the longevity-promoting function of FoxO TFs that has been established in experimental organisms may also be conserved in humans (Joshi et al., 2017). Therefore, understanding how FoxO TFs extend life span will likely lead to general insights into the aging process and new approaches to improve healthy aging.

The role of FoxO TFs in life span control was discovered in the nematode *Caenorhabditis elegans*, where the FoxO TF DAF-16 is the major target of the DAF-2 insulin-like signaling (ILS) pathway (Kenyon, 2010). DAF-2, the *C. elegans* ortholog of mammalian insulin and insulin-like growth factor (IGF) receptors, controls development and aging through a conserved PI3-kinase/Akt module that promotes the phosphorylation and nuclear export of DAF-16/FoxO (Kimura et al., 1997). In animals with reduced ILS, unphosphorylated DAF-16/FoxO translocates to the nucleus and regulates gene expression (Kimura et al., 1997; Murphy & Hu, 2005). Loss-of-function *daf-2/IGFR* mutations extend life span in a manner that is entirely dependent upon *daf-16/FoxO*, indicating that DAF-16/FoxO promotes life span extension in animals with reduced DAF-2 ILS (Kenyon et al., 1993). DAF-16/FoxO is also required for life span extension animals

lacking a germline as well as in some models of dietary restriction (Greer et al., 2007; Hsin & Kenyon, 1999).

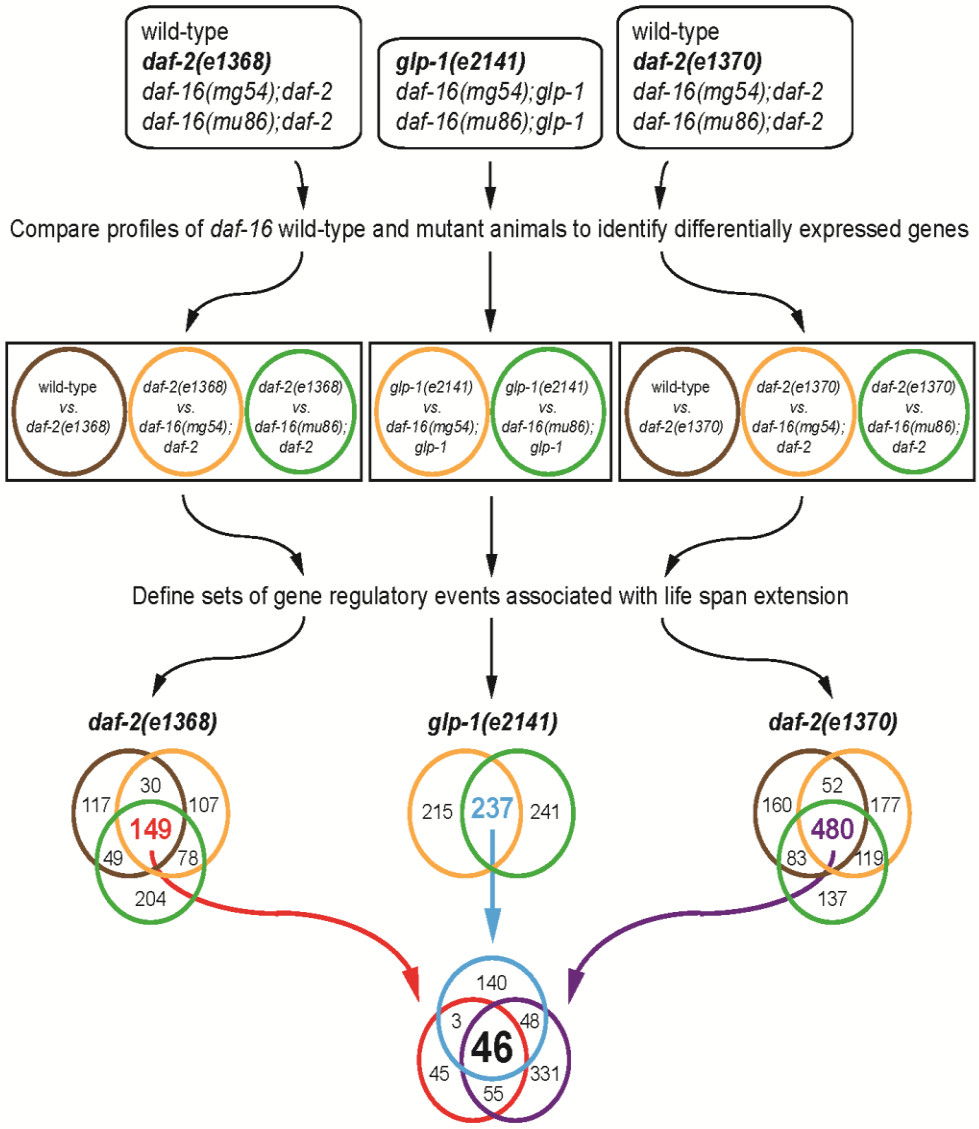
The mechanistic basis for life span extension by DAF-16/FoxO remains poorly understood. Expression profiling by multiple groups has identified thousands of DAF-16/FoxO-regulated genes (McElwee et al., 2003; McElwee et al., 2004; Murphy, 2006; Murphy et al., 2003). It is likely that multiple DAF-16/FoxO-dependent gene regulatory events contribute to DAF-16/FoxO-dependent longevity (Kenyon & Murphy, 2006). However, the majority of DAF-16/FoxO targets have not been interrogated for their influence on organismal longevity. Progress in this area has been impeded by the prodigious number of genes that are regulated by DAF-16/FoxO. By using filtering approaches to narrow the broad set of DAF-16/FoxO-regulated genes down to more tractable gene sets that are amenable to detailed functional analysis, several groups have succeeded in elucidating specific roles in life span control for a small number of DAF-16/FoxO target genes (Chen et al., 2015; Kaletsky et al., 2016; Lee et al., 2003; Tepper et al., 2013; Tullet et al., 2014).

Although the molecular requirements for DAF-16/FoxO regulation in the contexts of reduced ILS and germline ablation differ, we hypothesized that DAF-16/FoxO extends life span in both contexts by concordantly regulating a common set of target genes (Berman & Kenyon, 2006; Ghazi et al., 2009; Paradis et al., 1999; Paradis & Ruvkun, 1998). Previously reported expression profiles revealed little overlap in sets of DAF-16/FoxO target genes regulated by *daf-2/IGFR* and the germline (McCormick et al., 2012; Murphy et al., 2003). However, the interpretation of these studies is confounded by their use of different profiling platforms, which can be a source of variability in such experiments (Murphy, 2006). To clarify this issue, we used

whole transcriptome profiling to define sets of genes regulated by DAF-16/FoxO in *daf-2/IGFR* mutants and in germline-ablated animals.

Figure 4.1. Transcriptome profiling strategy for identifying DAF-16/FoxO targets associated with longevity (*dal* genes)

Figure 4.1. To delineate a set of DAF-16/FoxO target genes in the context of ILS we profiled *daf-2(e1368)* and *daf-2(e1370)* strains and identified genes that were differentially regulated in each *daf-2* strain relative to WT, and oppositely regulated in each *daf-16;daf-2* vs *daf-2* comparison. To delineate DAF-16/FoxO target genes in the context of germline ablation we identified genes that were differentially regulated in each *glp-1* vs *daf-16;glp-1* comparison. We then prioritized 46 *dal* genes that were regulated concordantly in all long-lived vs short-lived comparisons.



Results

A genetic filtering strategy to identify DAF-16/FoxO target genes associated with longevity

We previously performed whole transcriptome profiling in several *daf-16/FoxO* mutant backgrounds to define the roles of specific DAF-16/FoxO isoforms in the control of development and longevity (Chen et al., 2015). Here we employed a similar strategy to identify DAF-16/FoxO target genes in the contexts of reduced ILS and germline ablation (Figure 4.1; see Materials and Methods for details). To delineate the set of DAF-16/FoxO target genes in animals with reduced ILS, we identified genes that were differentially expressed in the *daf-2/IGFR* mutant *vs.* wild-type comparisons and differentially regulated in the opposite direction in the *daf-16;daf-2 vs. daf-2* comparisons. To increase the specificity of identifying genes regulated by ILS, we compared wild-type profiles to those of animals harboring either of two distinct *daf-2/IGFR* loss-of-function alleles: the strong Class II loss-of-function allele *e1370,* which is a missense mutation in the cytoplasmic tyrosine kinase domain, and the weaker Class I loss-of-function allele *e1368,* which is a missense mutation in the extracellular ligand binding domain (Gems et al., 1998; Kimura et al., 1997). To profile animals lacking a germline, we utilized the temperature-sensitive *glp-1(e2141)* mutant, which fails to develop a germline when raised at the restrictive temperature of 25°C (Arantes-Oliveira et al., 2002; Priess et al., 1987). Embryos harboring the *glp-1(e2141)* mutation were grown at 25°C to ablate the germline prior to RNA isolation. DAF-16/FoxO target genes were identified by comparing the expression profiles of *glp-1(e2141)* mutants to that of *daf-16;glp-1* double mutants. We did not compare profiles of germline-ablated animals with those of wild-type animals, as many differentially expressed genes in this comparison would be germline-specific and embryonic genes. To enrich sets of DAF-16/FoxO target genes for regulatory events that coincide with life span control, we used

two distinct *daf-16/FoxO* loss-of-function mutants: the *mu86* null allele (henceforth referred to as "*daf-16(null)"*and the *mg54* nonsense allele which abrogates the function of the *daf-16a* and *daf-16f* isoforms but does not affect the *daf-16b* isoform (henceforth referred to as "*daf-16(a/f)"*) (Lin et al., 1997; Ogg et al., 1997). We and others previously showed that *daf-16a* and *daf-16f* are the isoforms that promote longevity in the context of reduced ILS (Chen et al., 2015; Kwon et al., 2010). Therefore, we reasoned that genes specifically regulated by DAF-16A and/or DAF-16F would be more likely to play roles in life span control than DAF-16B-regulated genes. Life span assays on a subset of animals from each cohort profiled revealed the expected phenotypes (Figure 4.2).

A set of 46 dal (DAF-16/FoxO target associated with longevity) genes are concordantly regulated by DAF-16/FoxO in the contexts of reduced insulin-like signaling and germline ablation

To identify DAF-16/FoxO-dependent gene regulatory events that correlate with longevity, we collated sets of genes the regulation of which was influenced by both *daf-16(null)* and *daf-16(a/f)* alleles. This analysis yielded 149 genes in the *daf-2(e1368)* context, 480 genes in the *daf-2(e1370)* context, and 237 genes in the germline-ablated *glp-1(e2141)* context that were differently regulated in the wild-type and mutant backgrounds and differentially regulated in the opposite direction in both *daf-16(null) and daf-16(a/f)* mutants (Figure 4.1). Combining these gene sets revealed a set of 46 DAF-16/FoxO target genes that are concordantly regulated in all three contexts (Table 4.1). As these gene regulatory events are associated with the DAF-16/FoxO-dependent life span extension phenotype, we refer to these genes as *dal* (*D*AF-16/FoxO target *a*ssociated with *l*ongevity) genes. According to prior classifications of DAF-16/FoxO-regulated genes, 42 of 46

dal genes were Class I genes (induced in a DAF-16/FoxO-dependent manner), and four were Class II genes (repressed by DAF-16/FoxO) (Table 4.1) (Murphy et al., 2003; Tepper et al., 2013).

We compared our *dal* genes to a list of over three thousand DAF-16/FoxO-regulated genes that emerged from a meta-analysis of expression profiling experiments designed to identify consensus DAF-16/FoxO targets (Tepper et al., 2013). 39 of 42 Class I *dal* genes were identified as Class I DAF-16/FoxO targets by Tepper *et al.* (Table 4.1) (Tepper et al., 2013). Fourteen of the top 50 and six of the top ten consensus Class I DAF-16/FoxO targets identified by Tepper *et al.* are *dal* genes (Table 4.1)(Tepper et al., 2013). Three of four Class II *dal* genes were identified as Class II consensus targets by Tepper *et al.*, and two of these are among their top five Class II consensus targets (Table 4.1) (Tepper et al., 2013). Taken together, these data indicate that our filtering strategy has succeeded in prioritizing a small set of *bona fide* DAF-16/FoxO target genes.

Table 4.1. *dal* genes overlap with consensus DAF-16/FoxO targets

Gene	Annotation	Fold increase in *daf-2(e1370)* *vs.* wild-type	Fold decrease in *daf-16(mg54);daf-2* *vs. daf-2*	Tepper rank
dal-1/F56D6.9		>200	>200	
dal-2/T12D8.5		>200	>200	54
cest-1	carboxylesterase	200	>200	13
dal-3/Y6G8.9	F-box-associated domain	112	>200	
mtl-1	metallothionein	111	74	1
dal-4/Y39G8B.7	ShKT domain	79	87	294
dct-8		72	>200	445
dal-5/R05H10.7		59	91	442
dal-6/F15B9.6		48	95	158
dal-7/F21C10.11		36	172	366
sdz-24	OB-fold domain	24	175	134
lipl-2	triacylglycerol lipase	23	33	333
dal-8/C08E8.4	NADAR domain	22	25	21
dal-9/K03D3.2		21	12	186
dod-3		20	18	7
dlhd-1	dienelactone hydrolase	18	34	733
dal-10/C08F11.3		17	100	46
sod-3	superoxide dismutase	15	12	41
dal-11/Y20C6A.1	F-box, DUF38	14	60	209
dal-12/Y6G8.2	F-box, DUF38	14	60	15
lys-7	lysozyme	13	16	5
ftn-1	ferritin heavy chain	12	29	4
gst-29	prostaglandin D synthase	12	24	292
hen-1	LDL receptor motif	11	9	49
sru-40	serpentine receptor	9	>200	1071
glb-1	globin	7	8	97
spp-2	antimicrobial peptide	7	13	1421
dal-13/Y34F4.2	claudin-like tight junction protein	6	4	288
dal-14/M01B2.13	PHYHIPL ortholog	6	18	
btb-16	BTB/POZ domain	5	8	8
btb-17	BTB/POZ domain	5	8	58
cpg-7		4	6	316
cutl-24	zona pellucida domain	4	4	465
dal-15/Y38C1AA.6	RING/FYVE/PHD-type zinc finger	4	4	1116

ttr-44	transthyretin-like	4	3	38
icl-1	isocitrate lyase/malate synthase	4	6	-1598
dal-16/F48D6.4		4	2	2
hsp-12.6	α-crystallin/heat shock protein	3	3	123
hda-5	histone deacetylase	3	3	711
akt-2	Akt/Protein Kinase B	3	3	241
ttr-5	transthyretin-like	3	3	50
dal-17/Y2H9A.4	DUF713	2	3	5465
dal-18/K12B6.11		0.05	0.06	
irg-5	CUB-like domain	0.18	0.09	-5
dod-23		0.29	0.13	-1
srh-70	serpentine receptor	0.29	0.23	-1660

Table 4.1. List of DAF-16/FoxO targets associated with longevity. Fold changes in two right columns are based on sequencing read counts. Left column shows comparison to a meta-analysis which ranked DAF-16/FoxO targets from profiling experiments. Green boxes denote Class I DAF-16/FoxO target genes (upregulated in a DAF-16-dependent manner) that are in the top 50 consensus targets from the meta-analysis. Red boxes denote Class II DAF-16/FoxO target genes (DAF-16/FoxO dependent downregulated) that are in the top 50 consensus targets from the meta-analysis (Tepper et.al, 2013).

Figure 4.2. *daf-16a* and *f* isoforms are required for lifespan extension in *daf-2* mutants and germline ablated *glp-1* mutants

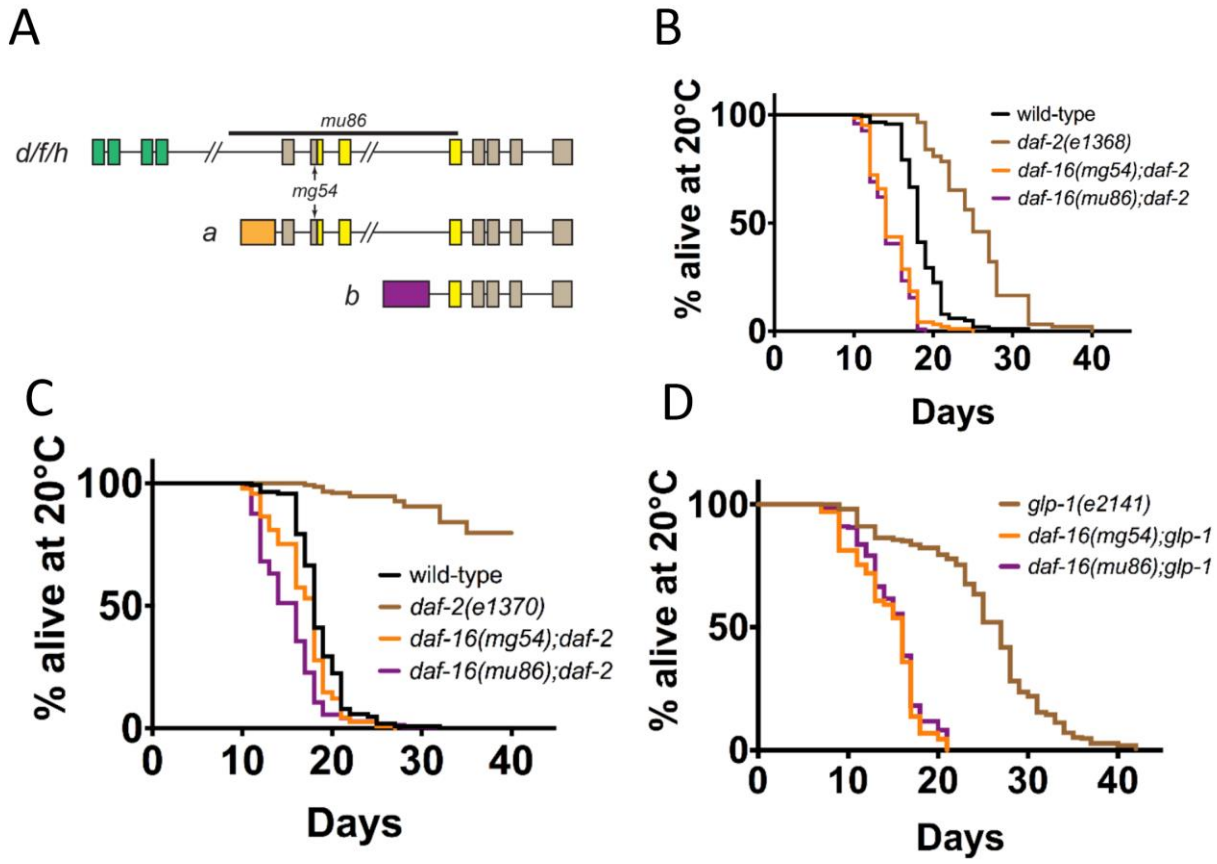


Figure 4.2. A. Schematic of the *daf-16* locus. Exons specific to isoform groups are labeled in green orange and purple, predicted DNA binding domain of DAF-16 is labeled in yellow. *daf-16(mu86)* is a deletion that effects all *daf-16* isoforms and is predicted to be a null allele, *daf-16(mg54)* is a premature stop mutation that affects the *daf-16a* and *daf-16f* isoform groups. Both alleles fully eliminate lifespan extension in B. *daf-2(e1368)*, C. *daf-2(e1370)* and D. *glp-1(e2141)* germline ablated animals.

The carboxylesterase family member CEST-1 is required for DAF-16/FoxO dependent lifespan extension

To identify *dal* genes that play roles in life span control, we obtained publicly available *C. elegans* strains harboring predicted strong loss-of-function mutations in Class I *dal* genes and performed life span assays in the context of reduced ILS. If a Class I *dal* gene (which is induced by DAF-16/FoxO)contributes significantly to DAF-16/FoxO-dependent life span extension, then reduction of *dal* gene activity should shorten life span specifically in the context of reduced ILS, where DAF-16/FoxO is active (Murphy et al., 2003). In our initial screen we subjected 23 *dal* mutants to life span assays after initiating *daf-2* RNAi to reduce ILS. Mutant strains in which *daf-2* RNAi extended life span to a significantly lesser degree than in the wild-type background were outcrossed three times prior to repeat life span assays.

After retesting, two *dal* mutants exhibited reduced life span extension by *daf-2* RNAi compared to wild-type animals. One mutant contained a loss-of-function mutation in *lipl-2,* which encodes a lipase previously implicated in lifespan regulation and response to fasting (Amrit et al., 2016; O'Rourke & Ruvkun, 2013). The other strain contained a mutation in *cest-1,* which encodes a member of the carboxylesterase family of enzymes that hydrolyze carboxyl esters to yield a carboxylic acid and an alcohol as products (Lian et al., 2017; Redinbo & Potter, 2005). Transcription of *cest-1* was confirmed to be robustly upregulated in *daf-2(e1370)* mutants in a *daf-16* dependent manner by qPCR (Figure 4.3). CEST-1 contains a putative signal peptide, a domain with homology to the carboxylesterase family of alpha/beta hydrolases, and a predicted

carboxyterminal transmembrane domain (Figure 4.4). Members of the carboxylesterase family have a conserved serine, glutamate, and histidine catalytic triad that is conserved CEST-1.

To verify our initial results, we tested the effect of *cest-1* mutation on the life spans of *daf-2* and *glp-1* mutants. We used the *cest-1(tm5130)* allele, which contains an out-of-frame deletion within the carboxylesterase domain that is predicted to introduce a premature stop codon 3' to the deletion (Figure 4.4). Since the protein predicted to be encoded by *cest-1(tm5130)* lacks a significant portion of the carboxylesterase domain, it is likely a null allele and will henceforth be designated "*cest-1(null).*" *cest-1(null)* suppressed the life span extension phenotype of two *daf-2* alleles: *e1368* (Figure 4.5A) and *m577* (Figure 4.5B). *cest-1(null)* did not significantly influence life span in the context of wild-type DAF-2 ILS, suggesting that the reduction in life span caused by *cest-1(null)* is not a consequence of general sickness. In contrast, *cest-1(null)* did not affect life span extension caused by *daf-2(e1370)* (Figure 4.5C) or germline ablation (Figure 4.5D). In these two contexts, the regulation of other DAF-16/FoxO target genes may suffice to compensate for loss of CEST-1 activity.

As the catalytic triad in carboxylesterases is conserved in CEST-1, we sought to determine the effect of specifically abrogating CEST-1 enzymatic activity on longevity by engineering a point mutation affecting one of the conserved putative catalytic residues into the *cest-1* genomic locus (See Figure 4.9 for alignment). We used CRISPR/Cas9-based genome editing to generate *cest-1(dp683),* which harbors a point mutation predicted to change serine 213 in wild-type CEST-1 to alanine (henceforth designated as "*cest-1(S213A)"*; Figure 4.4). this mutation has been shown to abolish enzymatic activity in human Ces1d (also known as Triacyl-glycerol hydrolase) (Alam et al., 2002). As observed with *cest-1(null), cest-1(S213A)* partially suppressed the life span

extension caused by *daf-2(e1368)* (Figure 4.5E). Intriguingly, in the context of wild-type DAF-2 ILS, *cest-1(S213A)* caused a modest extension in life span (Figure 4.5E).

Figure 4.3. *cest-1* is transcriptionally induced in a DAF-16/FoxO dependent manner in *daf-2(e1370)* mutants

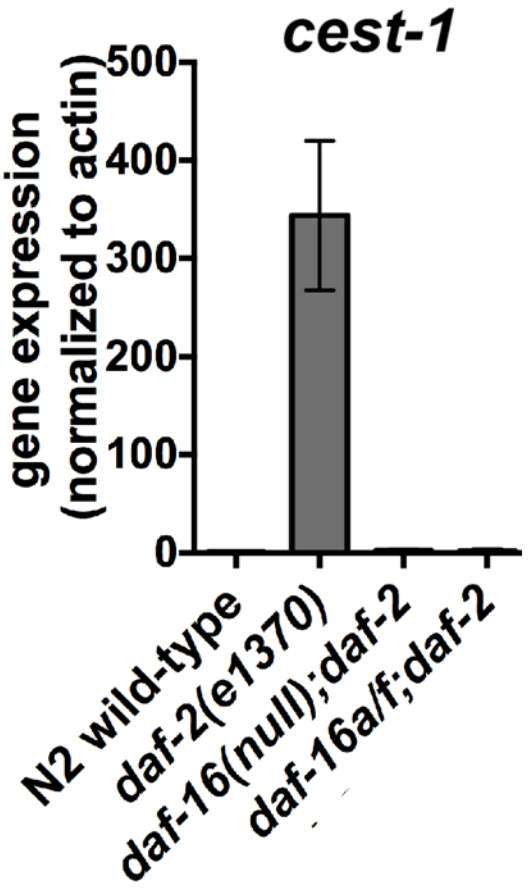


Figure 4.3**.** Quantitative-Real-Time-PCR measuring *cest-1* transcript levels in *daf-2(e1370), daf-16(null);daf-2* and *daf-16a/f;daf-2* mutants.

Figure 4.4. *cest-1* encodes a carboxylesterase family member
Figure 4.4. A. *cest-1* genomic locus. We utilize two *cest-1* alleles: *cest-1(tm5130)* is a predicted null allele, and *cest-1(dp683)* is a missense allele in which the conserved catalytic serine residue at position 213 is mutated to alanine. B. Domain structure of CEST-1 protein. Abbreviations: SP: signal peptide; TM: transmembrane domain.

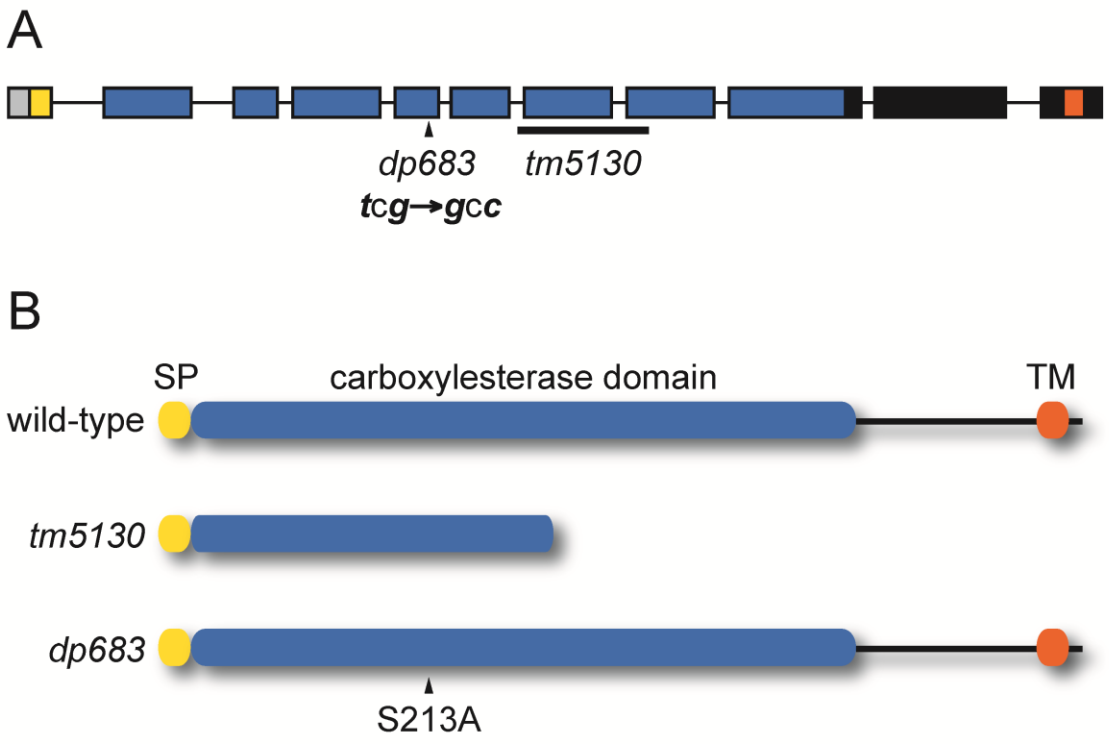
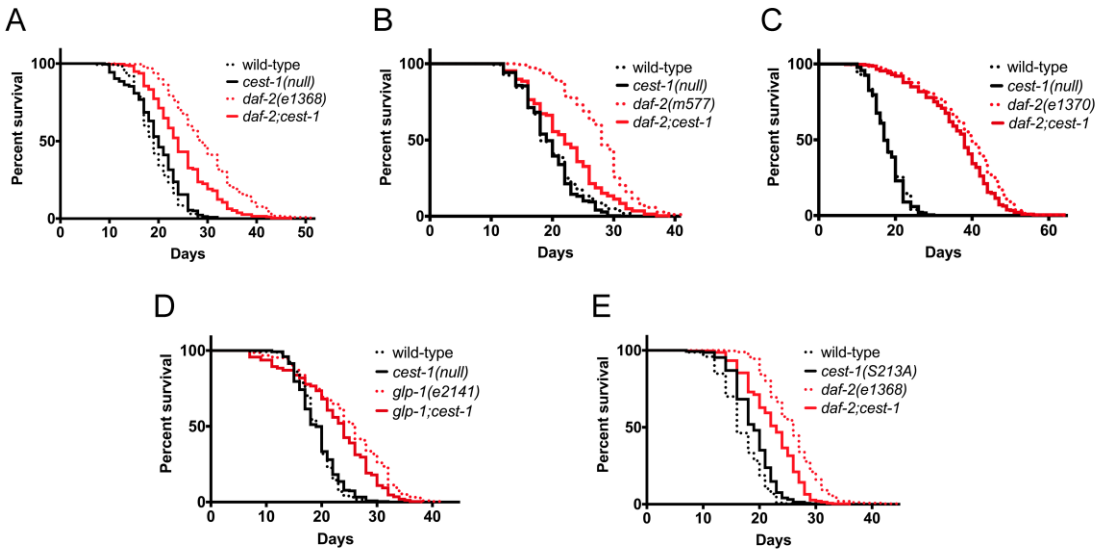


Figure 4.5. *cest-1* activity is required for lifespan extension in *daf-2*/IGFr mutants
Figure 4.5. Survival assays of *cest-1* mutants in long-lived backgrounds. A-B. *cest-1(tm5130)* suppresses lifespan extension phenotypes of *daf-2(e1368)* and *daf-2(m577)*. C-D. *cest-1(tm5130)* does not robustly suppress lifespan extension phenotypes of *daf-2(e1370)* or germline ablated *glp-1(e2141)* mutants. E. Targeted *cest-1* mutation converting the predicted catalytic serine to alanine suppresses the lifespan extension phenotype of a *daf-2(e1368)* mutant.



CEST-1::GFP is expressed in the intestine and localized at the luminal membrane

To determine the spatiotemporal expression pattern and subcellular localization of CEST-1, we generated transgenic animals expressing a CEST-1::GFP fusion protein under the control of the endogenous *cest-1* promoter and 3'UTR. We observed no visible GFP expression in two independent wild-type lines harboring the CEST-1::GFP transgene despite robust expression of the mCherry co-injection marker (Figure 4.7A). The CEST-1::GFP construct also had no effect on wild-type lifespan (Figure 4.6A).

Expression of the CEST-1::GFP transgene rescued lifespan extension in *daf-2(e1368);cest-1(tm5130)* animals, suggesting that CEST-1::GFP recapitulates endogenous CEST-1 expression and function in the tissues where it is required for lifespan extension (Figure 4.6A). Expression of CEST-1::GFP also enhanced the lifespan extension phenotypes of *daf-2(e1370)* but not *daf-2(e1368)* mutants (Figure 4.6A-B). Given the higher expression levels of DAF-16 targets, including *cest-1*, in *daf-2(e1370)* compared to *daf-2(e1368),* this is consistent with a model whereby high levels of *cest-1* expression can enhance lifespan extension of *daf-2* mutants. In *daf-2(e1368)* or *daf-2(e1370)* backgrounds we observed expression of CEST-1::GFP in the intestine, consistent with the expression pattern reported for wild-type *cest-1* transcripts in recently reported single-cell transcriptomic analyses (Blazie et al., 2017; Cao et al., 2017).

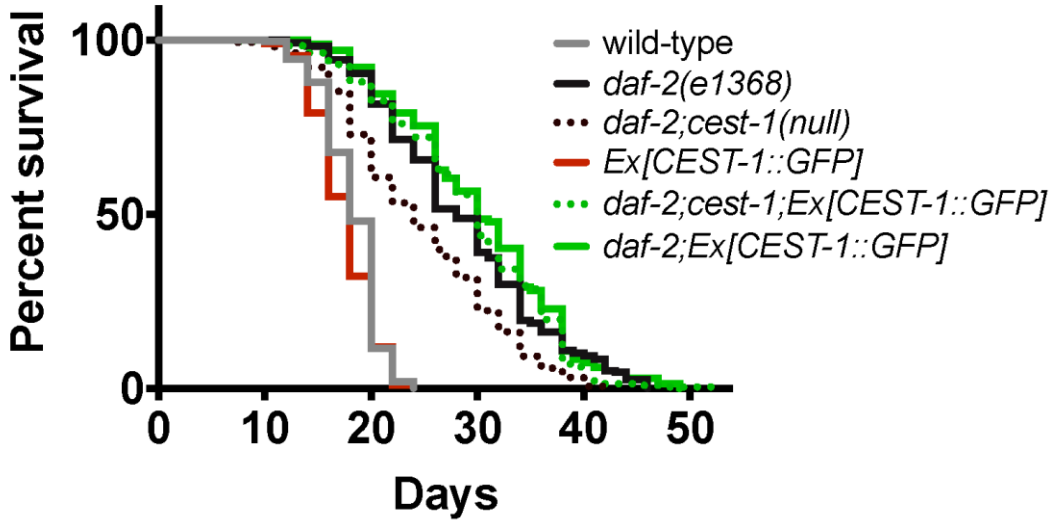
To assess the subcellular localization of CEST-1 we performed confocal microscopy on *daf-2(e1370)* animals expressing CEST-1::GFP (Figure 4.7). We observed that CEST-1::GFP was enriched at the luminal membrane of intestinal cells (4.7 B-E). Outside of the luminal membrane, CEST-1::GFP is present in punctae (Figure 4.7B). This expression pattern, along with analysis of the predicted CEST-1 protein sequence, is consistent with a model where CEST-1 is targeted to

the secretory pathway by its signal peptide, trafficked to the luminal membrane of the intestine, and is retained in the luminal membrane via it's transmembrane domain, with its catalytic domain facing into the intestinal lumen.

Figure 4.6. CEST-1::GFP rescues the lifespan phenotype of *daf-2(e1368);cest-1* mutants and enhances the lifespan extension phenotype of *daf-2(e1370)* mutants

Figure 4.6. Lifespan of strains expressing a C-terminal GFP tagged CEST-1 construct under its endogenous promoter with its endogenous 3'UTR and their non-transgenic siblings on A. wild-type, *daf-2(e1368);cest-1(tm5130)* and *daf-2(e1368)* backgrounds or B. the *daf-2(e1370)* background Aggregated data from three biological replicates. See Table 4.2 for summary data.

A



B

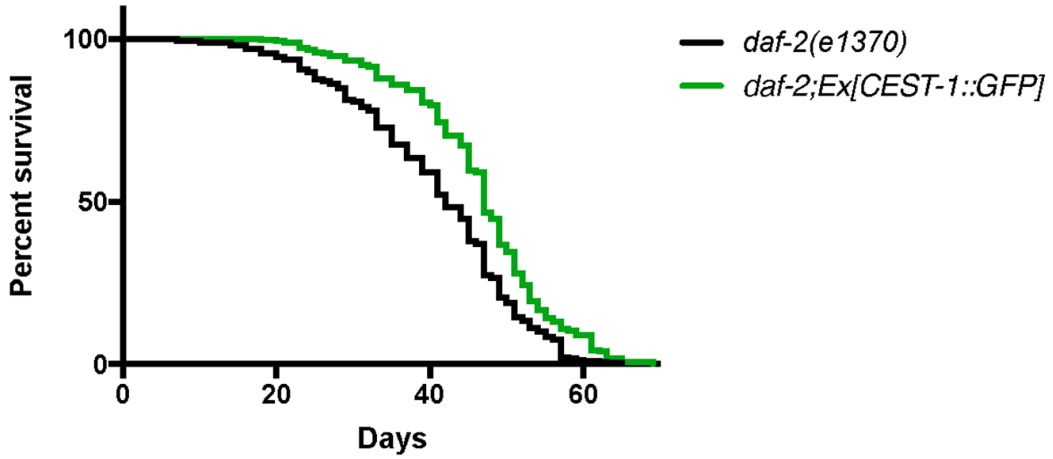
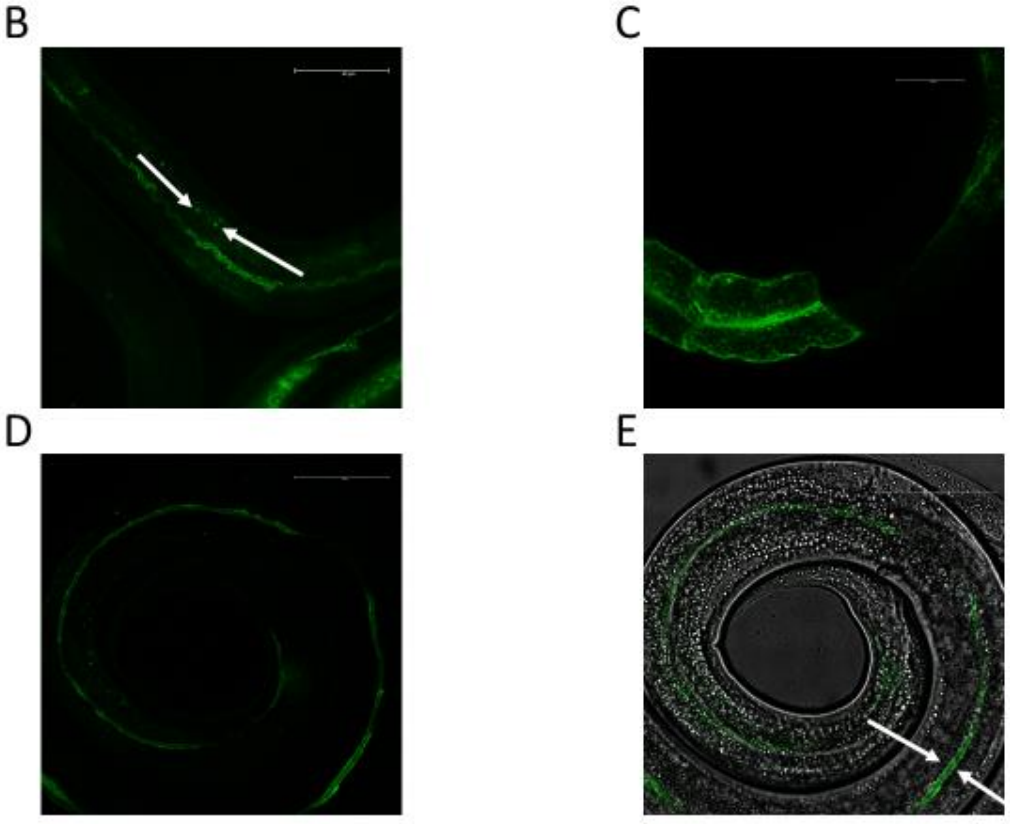


Figure 4.7. CEST-1::GFP is enriched at the intestinal lumen in *daf-2* mutants
Figure 4.7. A. Wild type animal harboring a transgene expressing CEST-1::GFP under its own promoter and mCherry co-injection marker expressed under the pharyngeal *myo-2* promoter. B-E. Confocal images of *daf-2(e1370)* animals expressing CEST-1::GFP under its endogenous promoter and 3'UTR. B. CEST-1::GFP is present in punctae with increased density near the lumen (white arrows) consistent with vesicular trafficking of CEST-1 to or from the membrane. C-E. CEST-1::GFP is present at the lumen, white arrows in panel E point to lumen .



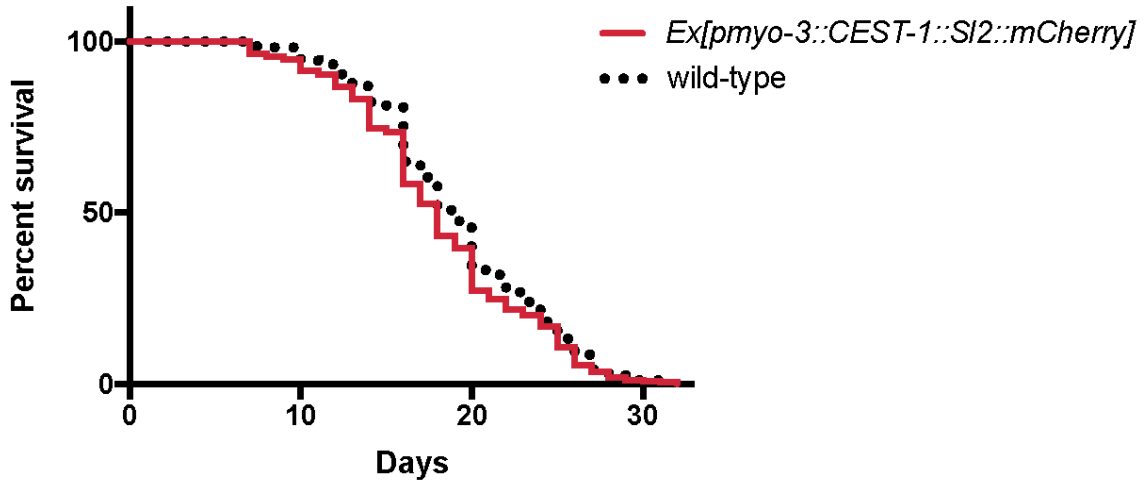
CEST-1 expression is sufficient to extend lifespan

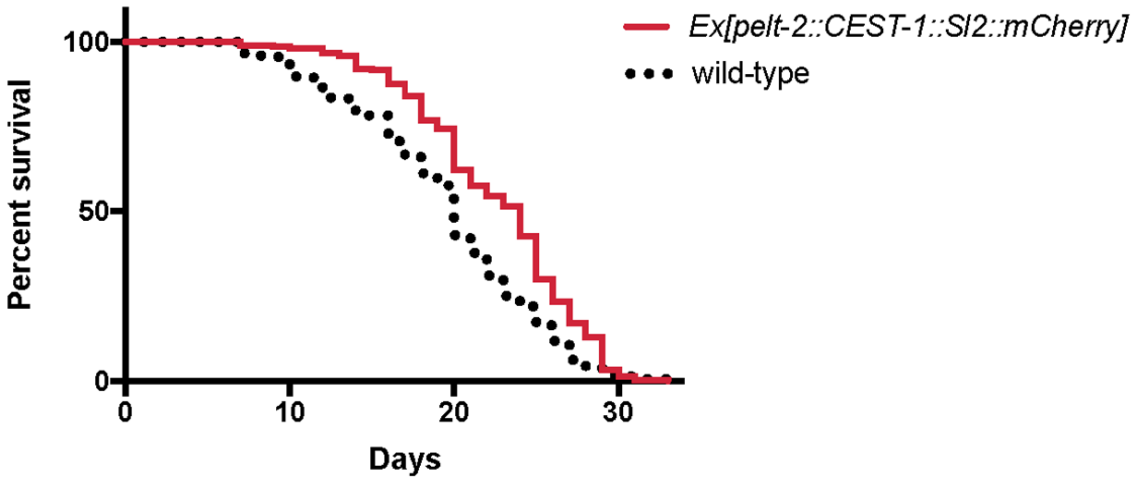
To test the hypothesis that expression of CEST-1 in the intestine is sufficient to extend lifespan, we generated transgenic lines expressing CEST-1 under promoters specific for the intestine (*pelt-2)* along with body wall muscle (*pmyo-3)* as a negative control. We observed that expression of CEST-1 in intestine was sufficient to slightly extend lifespan on a wild-type background, while expression in muscle did not extend lifespan. (Figure 4.8).

Figure 4.8. CEST-1 expression is sufficient to extend lifespan

Figure 4.8. A. Lifespan of *daf-2(e1370)* expressing CEST-1::GFP under its endogenous promoter and 3'UTR compared to non-transgenic siblings. B. Lifespan of wild-type with expression of CEST-1::SL2::mCherry in muscle (*pmyo3*) compared to non-transgenic siblings. C. Lifespan of wild-type with expression of CEST-1::SL2::mCherry in intestine (*pelt-2*).

A





B

Figure 4.9. Alignment of CEST-1 to human brain carboxylesterase

Figure 4.9. Comparative sequence analysis with human proteins reveals that CEST-1 is most homologous to brain carboxylesterase (hBr2). A. Alignment of CEST-1 and hBr2 coding sequences, predicted catalytic residues in bold B. CEST-1 protein coding sequence, putative carboxylesterase domain and transmembrane domain underlined, catalytic residues in bold.

A

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CEST-1 25   TIEGKILNISYSPLG-NQSATVFLGIPFVEPPIGDLRYRKPRPPKSWEGVLVTNEYKSAC  83
            T++GK+L    S  G  Q   VFLG+PF +PP+G LR+  P+  + W  V  T  Y   C
hBr2   28   TVQGKVLGKYVSLEGFAQPVAVFLGVPFAKPPLGPLRFAPPQAAEPWNFVKNTTSYPPMC  87

CEST-1 84   MSNAT---------KTYKNKFGGPISEDCLYLNVLTNEYCLENKNCSVMMIVHGGGYLTE  134
              +A             K+      SEDCLYLN+ T     +N    VM+ +HGGG +
hBr2   88   SQDAVGGQVLSELFTNRKDNIPLKFSEDCLYLNIYTPADLTKNSRLPVMVWIHGGGLVVG  147

CEST-1 135  SASTFNPEILINNFVGQGRNIVVVTFNYRLGLFSFGQFNGDRGDKNFGLYDMIESVNWVR  194
             AST++   L  +      N+VVVT  YRLG++ F     + G  N+G  D + ++ WV+
hBr2   148  GASTYDGLALSAH-----ENVVVVTIQYRLGIWGFFSTGDEHGRGNWGHLDQLAALRWVQ  202

CEST-1 195  REIENFGGNKNRITLAGHSAGASMIVAFTSSPLTKGLVHQQI-----IMSAPMTNMSKKS  249
              I NFGGN   +T+ G SAG   +     SPL K L H+ I      ++A +     K
hBr2   203  ENIANFGGNPGSVTIFGESAGGESVSVLVLSPLAKNLFHRAISESGVALTAALVKKDMKD  262

CEST-1 250  NFKGMTVMAQMVGCLSEEIGFNKLSEEQVENTYSCLRKKSAQQILDAQLWLLQNSTYFLG  309
              + + V A   GC S                  CLR+K+  ++L+  L L   +   LG
hBr2   263  TAQQIAVFA---GCKSTTSAV----------LVHCLRQKTEDELLEVSLKLKFFTLDLLG  309

CEST-1 310  AP---------PIDEHFLTDYPENLYASKSIYPINTLIGTTTLEVEESSYIID-----PA  355
             P          +D   L   P+ + A K    +  +IG   +  +E  +++      P
hBr2   310  DPRESYPFLPTVVDGVLLPKMPQEILAEKKFNSVPYIIG---INKQEFGWLLPMMMGYPL  366

CEST-1 356  FADKKVELLENLCDHIGYVLYEEPETFSKKCQKQYMNGNSS--------MNLSNEMEFYT  407
              DK  +   +      Y +   PE  +    ++Y+ G           +++  ++ F
hBr2   367  SEDKLDQKTASSLLWKSYPIANIPEELTPLASEKYLGGTDDPVKKKALFLDMLGDVVFGV  426

CEST-1 408  PAIDFADSHTSGNTKVFLYSYDYRGAGPAYDRYLEVRS--PHHSEDLIYVFGTH--RGIF  463
            P++  A  H       ++Y + Y    P++   ++ ++    H ++L  VFG    +G
hBr2   427  PSVTVARHHRDAGAPTYMYEFQYH---PSFSSDMKPQTVVGDHGDELFSVFGAPFLKGGA  483

CEST-1 464  APKDYIIEKIYSGMFADFVNFENPLPSGDQKWNQYTKENREHFLINFDKNFITPGMRDNY  523
            + ++  + K+   ++A+F    NP+  G   W  Y  + +E +L    +  I
hBr2   484  SEEEIRLSKMMMKLWANFARSGNPMGKGCSSWPAY--DQKEGYL----QIGIPTQPAQKL  537

CEST-1 524  YTEAYEFWSTVGKKSFKEEWSPS  546
             ++   FW+ +  K   E+ SP+
hBr2   538  KSKEMAFWTELLAKRAAEKLSPT  560
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B

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MLKPATVLLLIQMYVCKRVQLSSGTIEGKILNISYSPLGNQSATVFLGIPFVEPPIGDLRYRKPRPPKSWEGVLV

TNEYKSACMSNATKTYKNKFGGPISEDCLYLNVLTNEYCLENKNCSVMMIVHGGGYLTESASTFNPEILINNFVG

QGRNIVVVTFNYRLGLFSFGQFNGDRGDKNFGLYDMIESVNWVRREIENFGGNKNRITLAGHSAGASMIVAFTSS

PLTKGLVHQQIIMSAPMTNMSKKSNFKGMTVMAQMVGCLSEEIGFNKLSEEQVENTYSCLRKKSAQQILDAQLWL

LQNSTYFLGAPPIDEHFLTDYPENLYASKSIYPINTLIGTTTLEVEESSYIIDPAFADKKVELLENLCDHIGYVL

YEEPETFSKKCQKQYMNGNSSMNLSNEMEFYTPAIDFADSHTSGNTKVFLYSYDYRGAGPAYDRYLEVRSPHHSE

DLIYVFGTHRGIFAPKDYIIEKIYSGMFADFVNFENPLPSGDQKWNQYTKENREHFLINFDKNFITPGMRDNYYT

EAYEFWSTVGKKSFKEEWSPSLDTFTCALVISPLVSHMKQTTTAFDKTFEQTELLYKEEVNFLKREKLERTQELK

METKRRDKALRIQNRKNGLANKEITEGDEEDESSKLDILLIISAGTLFGGILYVTLPNVILQKRARDGYELLS
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Discussion

C. elegans has become a key model for understanding signal transduction pathways and cellular mechanisms that regulate organismal aging. Starting with seminal work demonstrating that *daf-2* loss-of-function mutations extend lifespan by activating DAF-16/FoxO, worm gerontologists have identified a variety of signal transduction pathways that regulate lifespan (Kenyon et al., 1993; Kenyon, 2010). These pathways converge on a small number of transcription factors whose activation can delay visible signs of aging and dramatically extend lifespan (Kenyon, 2010). Despite this body of work, and despite evidence that interventions such as calorie restriction and reduced insulin signaling extend lifespan in a conserved manner, the downstream transcriptional changes that are necessary and sufficient to delay aging remain poorly understood. This gap in knowledge is due in part to the sheer number of transcriptional changes caused by longevity promoting interventions such as DAF-16 activation. This has slowed down identification and detailed analysis of specific transcriptional targets that are critical for lifespan effects.

Here we have established a framework for identifying and characterizing genes that regulate lifespan downstream of DAF-16/FoxO activation. Reasoning that it would be most parsimonious if multiple contexts of DAF-16-dependent lifespan extension regulated longevity through the same transcriptional changes, we profiled three distinct long-lived strains and performed robust filtering for transcriptional changes that were both common to all three contexts and dependent upon the DAF-16 transcriptional isoforms that regulate lifespan. We identified a small set of *daf-16* targets associated with longevity (*dal* genes) and tested their role in lifespan regulation using publicly available mutants, avoiding caveats of RNAi-based candidate testing (Murphy, 2006). This

strategy enabled us to identify a novel DAF-16 target gene, CEST-1, as a new longevity regulator, and to follow up with a genetic characterization of *cest-1*.

CEST-1 encodes a previously unstudied member of the evolutionarily conserved carboxylesterase family of enzymes. CEST-1 has been independently identified as a consensus DAF-16 target gene in a meta-analysis of published profiling data but has not been tested for an effect on lifespan in published studies, highlighting the importance of unbiased genetic filtering strategies to prioritize targets for functional testing (Tepper et al., 2013).

In contrast to previous profiling studies, which usually focus on a single long-lived context, we tested a predicted *cest-1* null allele for suppression of lifespan in each of the three long-lived strains profiled (*daf-2(e1368)*, *daf-2(e1370)*, and *glp-1(e2141)*) as well as third *daf-2* mutant, *daf-2(m577)*. We found a robust genetic interaction between *cest-1* and the insulin signaling pathway, with *cest-1* mutation partially suppressing the lifespan increases caused by *daf-2(e1368)* and *daf-2(m577)*. Interestingly, *cest-1* mutation did not suppress the much larger lifespan increase of *daf-2(e1370)* mutants. We note that published work suggests that *daf-2(e1370)* and *(e1368)* mutants may have different causes of death, with longer lived *daf-2(e1370)* mutants being more resistant to colonization by intestinal bacteria than *daf-2(e1368)*, suggesting that CEST-1 might protect from bacterial colonization in *daf-2(e1368)* mutants but be dispensable in *daf-2(e1370)* mutants (Podshivalova et al., 2017). Alternatively, CEST-1 may be redundant with other DAF-16 targets within the large set of genes that are differentially regulated in *daf-2(e1370)* mutants but not *daf-2(e1368)* mutants.

cest-1 mutation did not suppress the lifespan extension of germline-ablated *glp-1* animals. This is consistent with previous work which has suggested that the requirements for long lifespan in *glp-1* animals may be different than in *daf-2*, with *glp-1* animals requiring *tcer-1, kri-1*, and *daf-12* for increased longevity while *daf-2* animals do not (Amrit et al., 2016; Berman & Kenyon, 2006; Ghazi et al., 2009). We also note that the unique demographic characteristics of *glp-1* animals, with a bi-modal lifespan distribution including a high rate of early death, may complicate identifying interactions with downstream mutations, such as *cest-1*, that primarily cause earlier onset of mortality. Overall, our data are consistent with a model where *cest-1* is necessary for full lifespan extension by reduced insulin signaling and suggest that understanding interactions between longevity effectors and multiple long-lived contexts in which they are induced will be important to provide insight into longevity mechanisms.

Consistent with our profiling data, we found that expression of a CEST-1::GFP translational fusion under its endogenous promoter is robustly induced by *daf-2(e1368)* and *daf-2(e1370)* mutation and is expressed in the intestine. The intestine has emerged as a major tissue regulating metabolism and aging in *C. elegans.* DAF-16 expression in intestine is sufficient to partially or fully rescue lifespan in *daf-2;daf-16* and *glp-1;daf-16* mutants, supporting the hypothesis that the intestine is the major site of DAF-16 action (Libina et al., 2003). Other well characterized downstream effector enzymes that promote increased longevity, including lipase family members downstream of DAF-16 and the flavin containing monooxygenase family member FMO-2 downstream of hypoxia and dietary restriction, are predominantly expressed in the intestine (Leiser et al., 2015; Wang et al., 2008). The intestine has diverse physiological roles including defense from pathogenic bacteria, digestion, metabolism, and adaptation to changing nutritional environments, that may be important for delayed mortality (McGhee, 2013).

Confocal microscopy shows that CEST-1::GFP is enriched at the luminal membrane of the intestine. Together with a predicted transmembrane domain in the CEST-1 protein, these data are consistent with a model where CEST-1 is targeted to the secretory pathway by its signal peptide, trafficked to the luminal membrane of the intestine, and is retained in the luminal membrane via its transmembrane domain, with its catalytic domain facing into the intestinal lumen. CRISPR/Cas9-mediated mutation of the predicted CEST-1 catalytic serine suppressed lifespan extension in a *daf-2(e1368)* mutant to a similar extent as *cest-1(null)*, suggesting that catalytic activity is required for CEST-1 to mediate lifespan extension.

Surprisingly, we found that expression of the CEST-1::GFP construct is sufficient to further extend the lifespan of long lived *daf-2(e1370)* mutants. We also observed a modest lifespan extension when CEST-1 was expressed under an intestine-specific promoter in wild-type animals, suggesting that CEST-1 activity is sufficient to promote longevity in the absence of reduced insulin signaling or increased DAF-16 activity. Overexpression of another DAF-16 target, the triglyceride lipase LIPL-4, extends lifespan through a mechanism that involves increasing levels of fatty acids which bind to and activate the transcription factors NHR-49 and NHR-80 (Folick et al., 2015). CEST-1 expression may similarly contribute to digestion of a dietary component that contributes to downstream signaling.

It remains unclear how CEST-1 activity promotes longevity. The localization of CEST-1::GFP at the luminal membrane is consistent with models in which CEST-1 is situated upstream in metabolic changes that promote longevity downstream of DAF-16/FoxO. For example, CEST-1 might function to aid in digestion of beneficial metabolites, such as precursors to the longevity extending polyunsaturated fatty acid species that are enriched in LIPL-4 (Folick et al., 2015;

O'Rourke et al., 2013). Alternatively, CEST-1 might function to break down toxic xenobiotics in the gut; the slight lifespan extension observed in wild-type animals expressing a predicted catalytically inactive CEST-1 protein is consistent with a model in which this protein is binding to and sequestering a toxic metabolite. Ultimately unbiased metabolomic studies may be of use in generating hypotheses about CEST-1 function by identifying CEST-1 substrates (Edison & Schroeder, 2010).

CEST-1 has sequence homology to mammalian esterases including members of the mammalian carboxylesterase (CES) superfamily which includes human CES-1-5 and members of the murine *Ces-1,2,3,4a,*and*5a* families (Lian et al., 2017). Mammalian carboxylesterases are expressed in intestine, liver, and adipose tissue and, in addition to detoxifying xenobiotics, hydrolyze endogenous lipids and other substrates including acetylcholine (Lian et al., 2017). Mouse models in which *Ces* family members have been knocked out suggest that these enzymes regulate obesity, energy expenditure, hepatic steatosis, and insulin sensitivity, suggesting that interactions between insulin signaling and carboxylesterase family members may be conserved and relevant to mammalian disease (Ko et al., 2009; Lian et al., 2017; Quiroga et al., 2012; Redinbo & Potter, 2005) . Published work has singled out the carboxylesterase family as one of a small number of gene families that are under selection during evolution of changes in vertebrate lifespan-suggesting that carboxylesterases could be conserved regulators of aging (Harel et al., 2015). To our knowledge this is the first functional study of a carboxylesterase family member in the regulation of lifespan.

Overall our study has established a novel method of enriching for DAF-16 target genes that are involved in longevity and characterized a previously unstudied member of the carboxylesterase

superfamily that is both necessary for lifespan extension in the context of reduced insulin signaling, and sufficient to extend the lifespan of wild type and *daf-2* mutant animals. Future studies will establish whether CEST-1 interacts with other contexts of increased lifespan and explore potentially conserved downstream mechanisms through which CEST-1 contributes to lifespan extension.

Materials and Methods

C. elegans strains and maintenance

Animals were maintained at 15°C on nematode growth media NGM seeded with *Escherichia coli*. strain OP50. Compound mutants were constructed using standard genetic techniques. Genotypes were confirmed by using PCR followed by restriction digestion to detect restriction fragment length polymorphisms or using three primer PCR assays to detect deletions based on product size. Percival incubators were used for maintenance and lifespan assays.

RNA isolation

Lifespans were performed on all strains profiled to confirm the expected phenotypes. The remaining animals from populations raised for life span assays were washed twice in M9 buffer. Total RNA was isolated using TRIzol reagent (Invitrogen) and purified using a RNeasy kit (QIAGEN Inc., Valencia, CA) according to manufacturers' instructions.

Whole transcriptome profiling (RNA-Seq)

Transcriptome profiling was performed in collaboration with the University of Michigan sequencing core. Five experimental replicates were used for each genotype. We used the Tuxedo

Suite for alignment, differential expression analysis, and post-analysis diagnostics. Gene expression output comprised eight comparisons in three groups: (1) *daf-2(e1370)* compared to wild-type, *daf-16(mu86);daf-2(e1370)*, and *daf-16(mg54);daf-2(e1370)*, (2) *daf-2(e1368)* compared to wild-type, *daf-16(mu86);daf-2(e1368)*, and *daf-16(mg54);daf-2(e1368)*, and (3) *glp-1(e2141)* compared to *daf-16(mu86);glp-1* and *daf-16(mg54);glp-1*. We defined DAF-16 targets in the *daf-2* context as meeting all of the following criteria: (1) test status = "OK", (2) fold change (FC) $\geq \pm 1.5$ for wild-type *vs. daf-2*, (3) FC ≥ 1.5 in the opposite direction as wild-type for both *daf-2 vs. daf-16(mu86);daf-2* and *daf-2 vs. daf-16a/f(mg54);daf-2*, and (4) FDR < 0.05 for all three comparisons. The criteria for *glp-1* animals was similar except there was no wild-type comparison. We noted that many genes previously validated as DAF-16/FoxO targets did not meet our FDR criteria, and that RNA-seq is biased against short, low-expressed genes even if normalized for length. Therefore, we also included genes that did not meet the FDR requirement but did fulfill a more stringent FC > 3 criterion for all comparisons. Data are available at GEO with the accession number GSE111338.

Quantitative Real-Time reverse transcriptase PCR

cDNA was synthesized using a SuperScript® III Reverse Transcriptase kit and random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was performed in triplicate using *Power* SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) and a Mastercycler® ep *realplex* thermal cycler (Eppendorf North America, Westbury, NY). 10ng of cDNA was used as a template in a 15µl reaction volume. Relative expression levels and technical error were determined by the $\Delta\Delta 2C_t$ method. Gene expression levels were normalized to actin (*act-1*), and the ratio of expression

relative to *act-1* was then compared to the same ratio in N2 Bristol wild-type. Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA) using the paired ratio *t*-test.

Life span assays

Lifespans were performed as described previously with minor modifications (Chen et al., 2015). Animals derived from a synchronized 6-hour egg lay were grown at 15°C until the L4 larval stage to prevent any dauer formation, transferred to 20°C until day one of adulthood, then placed on plates containing 25ug/ml 5'-fluoro-2'-deoxyuridine (FUDR) to prevent progeny production and 0.001% nystatin to inhibit fungal growth. For lifespans including strains harboring *CEST-1::GFP* transgenes, animals were grown at 20°C from egg, which was determined empirically to increase visible expression of the GFP transgenes relative to culture at 15°C, potentially due to the temperature sensitivity of *daf-2* mutants, and animals with visible expression of the transgene were selected under a fluorescent dissecting scope at 48 hours post egg lay for inclusion in the assay. *glp-1* mutant animals were raised at 25°C for 48 hours from egg lay to ablate the germline and then switched to 20°C. Animals were scored as dead if they did not respond to being touched with a platinum wire pick, and animals with ruptured vulvas were censored. Statistical significance was assessed using the standard chi-square-based log rank test in GraphPad Prism.

Transgenic strain construction

CEST-1::GFP constructs were generated by fusing the CEST-1 coding sequence and a 513 bp *cest-1* promoter to GFP, followed by the CEST-1 3'UTR using overlap extension PCR and cloned into the TOPOXL vector, then injected along with *pmyo-2::mCherry::unc54utr* (pJK343) as a co-injection marker. To generate the polycistronic tissue-specific overexpression constructs,

intestinal (*elt-2*) and muscle (*myo-3*) specific promoters were fused to CEST-1 and the intercistronic genomic fragment between *gpd-2* and *gpd-3*, followed by *mCherry::unc-54utr* and injected as linear PCR products. All constructs were verified by Sanger sequencing prior to injection.

CRISPR/CAS9 based gene editing

cest-1(dp683) was generated using recombinant crRNA and tracrRNA (Dharmacon), Cas9 (PNABio) and a 100bp repair oligo (IDT) and injected with a *dpy-10* co-CRISPR marker as described previously (Paix et al., 2015).

Sequence of the *cest-1(dp683)* repair oligo is

5' GAAATTGAAAACTTTGGAGGAAATAAAAACAGAATTACATTGGCAGGGCATGCC

GCTGGAGCAAGTATGATAGTAGCGGTAGGTCACATAAATGATACATTTTTG 3'

Sequence of the 20-nucleotide gene specific sequence in the *cest-1(dp683)* crRNA is

5' ACCTACCGCTACTATCATAC 3'

Confocal microscopy

Animals were immobilized with 1 mg/ml Tricane (ethyl 3-aminobenzoate methanesulfonate salt) and 0.1mg/ml of tetramisole hydrochloride (TMHC) dissolved in M9 buffer as described previously (Green et al., 2011). Animals were mounted on slides layered with a thin 3% agarose in H_2O pad and images were captured on a Leica inverted SP5X Confocal microscope using LAS AF software.

Table 4.2. Summary data from lifespans

Table 4.2. Survival curves shown in figures ae comprised of aggregated from three biological replicates. Table (next 4 pages) includes median number of deaths, number censored due to rupture, and statistical comparisons by log rank test for individual lifespans and aggregated lifespans.

Replicate number. * If aggregate shown in figure	genotype	deaths (censored)	median survival (days)	P value (Log-rank)	P value compared to
1	*WT*	84(11)	17		
1	*cest-1(tm5130)*	48(1)	17		
1	*daf-2(e1368)*	74(1)	24		
1	*daf-2;cest-1*	82(1)	20	<.001	*daf-2*
2	*WT*	83(1)	20		
2	*cest-1(tm5130)*	90(11)	20		
2	*daf-2(e1368)*	76(4)	35		
2	*daf-2;cest-1*	101(0)	28	<.001	*daf-2*
3	*WT*	98(7)	21		
3	*cest-1(tm5130)*	82(0)	23		
3	*daf-2(e1368)*	85(12)	30		
3	*daf-2;cest-1*	84(3)	24	<.001	*daf-2*
*	*WT*	265(19)	19		
*	*cest-1(tm5130)*	220(12)	20		
*	*daf-2(e1368)*	236(17)	29		
*	*daf-2;cest-1*	267(4)	24	<.0001	*daf-2*
1	*WT*	96(8)	23		
1	*cest-1(tm5130)*	110(9)	23		
1	*daf-2(m577)*	114(5)	31		
1	*daf-2;cest-1*	77(5)	25	<.0001	*daf-2*
2	*WT*	134(12)	21		
2	*cest-1(tm5130)*	123(1)	19		
2	*daf-2(m577)*	97(6)	27		
2	*daf-2;cest-1*	80(16)	23	<.0001	*daf-2*
3	*WT*	117(3)	18		
3	*cest-1(tm5130)*	106(0)	18		
3	*daf-2(m577)*	125(5)	28		
3	*daf-2;cest-1*	110(11)	20	<.0001	*daf-2*
*	*WT*	330(14)	18		
*	*cest-1(tm5130)*	322(9)	19		
*	*daf-2(m577)*	364(15)	28		
*	*daf-2;cest-1*	297(27)	22	<.0001	*daf-2*
1	*WT*	94(14)	17		
1	*cest-1(tm5130)*	89(13)	17		
1	*daf-2(e1370)*	92(11)	43		
1	*daf-2;cest-1*	98(4)	39	0.18	*daf-2*
2	*WT*	71(12)	19		
2	*cest-1(tm5130)*	77(4)	18		
2	*daf-2(e1370)*	59(3)	38		
2	*daf-2;cest-1*	83(1)	34	0.009	*daf-2*
3	*WT*	78(4)	18		

Replicate number. * If aggregate shown in figure	genotype	deaths (censored)	median survival (days)	P value (Log-rank)	P value compared to
3	*cest-1(tm5130)*	74(2)	20		
3	*daf-2(e1370)*	87(1)	40		
3	*daf-2;cest-1*	98(1)	38	<.0001	*daf-2*
*	*WT*	243(30)	18		
*	*cest-1(tm5130)*	240(19)	17		
*	*daf-2(e1370)*	238(15)	40		
*	*daf-2;cest-1*	279(6)	38	0.0004	*daf-2*
1	*WT*	78(0)	20		
1	*cest-1(tm5130)*	83(4)	17		
1	*glp-1(e2141)*	78(6)	24		
1	*glp-1;cest-1*	112(4)	24	0.08	*glp-1*
2	*WT*	69(4)	21		
2	*cest-1(tm5130)*	60(0)	17		
2	*glp-1(e2141)*	65(34)	26		
2	*glp-1;cest-1*	85(27)	21	0.01	*glp-1*
3	*WT*	101(0)	18		
3	*cest-1(tm5130)*	100(1)	21		
3	*glp-1(e2141)*	91(8)	26		
3	*glp-1;cest-1*	66(8)	24	0.03	*glp-1*
*	*WT*	248(4)	19		
*	*cest-1(tm5130)*	243(5)	19		
*	*glp-1(e2141)*	234(47)	26		
*	*glp-1;cest-1*	263(39)	24	<0.001	*glp-1*
1	*WT*	95(5)	18		
1	*cest-1(dp683)*	102(1)	21		
1	*daf-2(e1368)*	100(2)	27		
1	*daf-2;cest-1*	92(0)	25	<.0001	*daf-2*
2	*WT*	64(4)	18		
2	*cest-1(dp683)*	97(2)	20		
2	*daf-2(e1368)*	92(8)	24		
2	*daf-2;cest-1*	106(2)	22	<.0001	*daf-2*
3	*WT*	59(18)	16		
3	*cest-1(dp683)*	94(6)	16		
3	*daf-2(e1368)*	66(12)	24		
3	*daf-2;cest-1*	98(4)	20	<.0001	*daf-2*
*	*WT*	218(27)	16		
*	*cest-1(dp683)*	293(9)	19		
*	*daf-2(e1368)*	258(22)	26		
*	*daf-2;cest-1*	296(6)	23	<.0001	*daf-2*
1	*WT*	106(7)	18		
1	*CEST-1::GFP*	115(7)	16		

Replicate number. * If aggregate shown in figure	genotype	deaths (censored)	median survival (days)	P value (Log-rank)	P value compared to
1	*daf-2;cest-1(tm5130)*	111(1)	24		
1	*daf-2;cest-1;CEST-1::GFP*	105(4)	30	<.0001	*daf-2;cest-1*
1	*daf-2*	114(3)	32		
1	*daf-2;CEST-1::GFP*	95(4)	30	0.91	*daf-2*
2	*WT*	101(0)	24		
2	*CEST-1::GFP*	91(1)	22		
2	*daf-2;cest-1(tm5130)*	87(1)	28	<.0001	*daf-2;cest-1*
2	*daf-2;cest-1;CEST-1::GFP*	69(7)	32		
2	*daf-2*	75(3)	33		
2	*daf-2;CEST-1::GFP*	80(6)	32	0.03	*daf-2*
3	*WT*	74(4)	20		
3	*CEST-1::GFP*	89(1)	20		
3	*daf-2;cest-1(tm5130)*	94(4)	22		
3	*daf-2;cest-1;CEST-1::GFP*	81(1)	30	<.0001	*daf-2;cest-1*
3	*daf-2*	66(5)	28		
3	*daf-2;CEST-1::GFP*	93(7)	28	0.83	*daf-2*
*	*WT*	281(11)	20		
*	*CEST-1::GFP*	295(9)	19		
*	*daf-2;cest-1(tm5130)*	292(6)	24		
*	*daf-2;cest-1;CEST-1::GFP*	255(12)	30	<.0001	*daf-2;cest-1*
*	*daf-2*	255(11)	30		
*	*daf-2;CEST-1::GFP*	268(17)	30	0.05	*daf-2*
1	*daf-2;cest-1(tm5130)*	92(3)	22		
1	*daf-2;cest-1;CEST-1::GFP*	56(9)	27	<0.001	*daf-2*
1	*daf-2*	71(4)	24		
1	*daf-2;CEST-1::GFP*	54(5)	30	0.0003	*daf-2*
2	*WT*	97(19)	20		
2	*CEST-1::GFP*	103(8)	16		
2	*daf-2;cest-1(tm5130)*	99(1)	22	<0.0001	*daf-2;cest-1*
2	*daf-2;cest-1;CEST-1::GFP*	90(3)	32		
2	*daf-2*	115(5)	26		
2	*daf-2;CEST-1::GFP*	92(16)	30	0.02	*daf-2*
3	*WT*	106(0)	18		
3	*CEST-1::GFP*	92(1)	18		
3	*daf-2;cest-1(tm5130)*	124(2)	30		
3	*daf-2;cest-1;CEST-1::GFP*	85(3)	32	0.02	*daf-2;cest-1*
3	*daf-2*	92(1)	34		
3	*daf-2;CEST-1::GFP*	70(2)	33	0.14	*daf-2*
*	*WT*	203(19)	18		
*	*CEST-1::GFP*	195(9)	18		
*	*daf-2;cest-1(tm5130)*	315(6)	24		

Replicate number. * If aggregate shown in figure		genotype	deaths (censored)	median survival (days)	P value (Log-rank)	P value compared to
*		*daf-2;cest-1;CEST-1::GFP*	231(15)	30	<0.0001	*daf-2*
*		*daf-2*	278(10)	28		
*		*daf-2;CEST-1::GFP*	216(30)	30	0.03	*daf-2*
	1	*daf-2(e1370)*	124(1)	41		
	1	*daf-2;CEST-1::GFP*	88(6)	45	0.0003	*daf-2*
	2	*WT*	100(3)	18		
	2	*daf-2(e1370)*	99(2)	41		
	2	*daf-2;CEST-1::GFP*	91(8)	47	0.0066	*daf-2*
	3	*WT*	120(4)	20		
	3	*CEST-1::GFP*	99(9)	20		
	3	*daf-2(e1370)*	115(0)	47		
	3	*daf-2;CEST-1::GFP*	105(2)	51	<0.0001	*daf-2*
*		*daf-2(e1370)*	338(3)	42		
*		*CEST-1::GFP*	284(16)	47	<0.0001	*daf-2*
	1	*WT*	87(0)	18		
	1	*pmyo-3::CEST-1*	98(0)	18	0.74	*WT*
	2	*WT*	74(3)	20		
	2	*pmyo-3::CEST-1*	99(2)	20	0.36	*WT*
	3	*WT*	68(0)	19		
	3	*pmyo-3::CEST-1*	74(0)	19	0.08	*WT*
*		*WT*	271(2)	18		
*		*pmyo-3::CEST-1*	229(3)	19	0.39	*WT*
(line2)	1	*WT*	94(0)	20		
	1	*pmyo-3::CEST-1*	94(0)	16	<0.0001	*WT*
	2	*WT*	90(3)	21		
	2	*pmyo-3::CEST-1*	102(3)	23	0.17	*WT*
	3	*WT*	75(6)	17		
	3	*pmyo-3::CEST-1*	85(1)	17	0.67	*WT*
*		*WT*	281(4)	19		
*		*pmyo-3::CEST-1*	259(9)	18	0.08	*WT*
	1	*WT*	88(3)	16		
	1	*pelt-2::CEST-1*	80(0)	20	<0.0001	*WT*
	2	*WT*	104(3)	23		
	2	*pelt-2::CEST-1*	120(2)	25	0.001	*WT*
	3	*WT*	95(1)	21		
	3	*pelt-2::CEST-1*	88(2)	23	0.0007	*WT*
*		*WT*	287(7)	20		
*		*pelt-2::CEST-1*	288(4)	24	<0.0001	*WT*

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Chapter 5 Conclusions

Overview

This dissertation started with the goal of identifying both novel mechanisms of DAF-16/FoxO regulation, and critical genes that work downstream of DAF-16/FoxO activity to regulate lifespan. In Chapter Two we used an unbiased forward genetics approach that was intended to identify novel regulators of DAF-16/FoxO. We unexpectedly identified a novel mutation *lin-14* that regulates the developmental decision to enter the dauer diapause and may act downstream of or in parallel to insulin-signaling and DAF-16/FoxO. In Chapter 4 we used an unbiased transcriptome profiling approach to identify DAF-16/FoxO target genes that are associated with longevity in multiple long-lived contexts. We then followed this up with a robust genetic characterization of a previously unstudied gene, *cest-1*, that contributes to longevity in the context of reduced insulin signaling. Both lines of inquiry have raised interesting questions for future study.

A novel mutation in *lin-14* regulates dauer entry

In order to activate a transcriptional program that delays aging, DAF-16/FoxO must be present in the nucleus of the cell and must bind to regulatory elements through which it controls the expression of downstream target genes. Many studies have established different molecular mechanisms that regulate the localization of DAF-16/FoxO; however, comparatively little is known about the requirements for full activity of nuclear DAF-16/FoxO. In addition to regulating lifespan, increased DAF-16 FoxO activity promotes developmental arrest in the dauer diapause: thus, screening for mutations that make worms more or less likely to enter dauer can identify new mechanisms of DAF-16/FoxO regulation. We had previously characterized EAK-7, a conserved plasma membrane protein that acts to inhibit activity of nuclear DAF-16/FoxO; we then attempted to identify molecules that act downstream of EAK-7 to regulate nuclear DAF-16/FoxO activity by performing a forward genetic screen for mutations that suppress the dauer constitutive phenotype of *eak-7;akt-1* compound mutants (Alam et al., 2010; Delaney et al., 2017; Dumas et al., 2013).

Forward genetics is a powerful tool which enables us to "ask the animals a question" and obtain an "answer" without guessing in advance what that answer might be. It frequently produces unexpected results. In this case our forward genetic screen identified a novel allele of *lin-14*, a well characterized regulator of the sequence in which early developmental events occur, that suppressed *eak-7;akt-1* dauer arrest. Interestingly, the *lin-14(dp69)* mutation we discovered was phenotypically distinct from strong *lin-14* loss-of-function alleles identified in screens for heterochronic phenotypes, both in the sense

that it lacked characteristic *lin-14* heterochronic phenotypes observed during non-dauer development and in the sense that it suppressed dauer-constitutive phenotypes rather than affecting the timing of dauer entry. Nonetheless both molecular analysis of the *lin-14* transcript and the observation that the *lin-14(dp69)* dauer suppression phenotype was phenocopied by *lin-14* RNAi are most consistent with the hypothesis that *lin-14(dp69)* is a novel class of *lin-14* reduction-of-function allele. *lin-14(dp69)* and *lin-14* RNAi might affect activity of the *lin-14b* isoform, since mutations that affect *lin-14b* also have phenotypes that are entirely different from *lin-14* null phenotypes and affect the second larval stage, which can progress to either a dauer or reproductive L3 during the dauer life history. Alternatively, *lin-14(dp69)* might have neomorphic or change-of-function effects, for example altering the expression pattern of the LIN-14 protein. Genetic analysis has suggested that *lin-14(dp69)* and *lin-14* RNAi can suppress the DAF-16/FoxO-independent dauer constitutive phenotype of *daf-7*/TGF-β mutants, indicating that *lin-14(dp69)* may influence dauer arrest independently of DAF-16/FoxO. Studies in the literature suggest that *lin-14* interacts genetically with *daf-12* which encodes a transcription factor that acts downstream of DAF-16/FoxO to influence the dauer decision. Other heterochronic genes regulate dauer through interactions with *daf-12*. These data suggest that *lin-14(dp69)* and *lin-14* RNAi could impinge upon mechanisms that integrate regulation of dauer entry with regulation of developmental timing.

Additional experiments could help to clarify the interactions between *lin-14(dp69)* and known regulators of the dauer decision. We could determine whether *lin-14(dp69)* and *lin-14* RNAi can singly or additively suppress the Daf-c phenotypes of *daf-12*/NHR ligand binding domain mutants, mutants defective in the biosynthesis of DAF-12 ligands,

and mutants in *lin-42*/period which acts in the heterochronic pathway and regulates the dauer decision through direct binding to DAF-12 If *lin-14(dp69)* and *lin-14* RNAi can suppress dauer arrest in dafachronic acid pathway mutants but not *lin-42*/per mutants, this might suggest that *lin-14* acts in a pathway with *lin-42*/per to regulate DAF-12 activity. Since *lin-14(dp69)* and *lin-14* RNAi are not null alleles and we cannot do formal genetic epistasis studies using them, we would need to bolster such inferences with molecular data, for example by determining whether *lin-14* RNAi or *lin-14(dp69)* affected expression of LIN-42/period protein.

We could also test the hypothesis that *lin-14(dp69)* and *lin-14*RNAi affect LIN-14 protein levels. Andrew Fire's lab has generated single copy LIN-14::GFP translational fusions at the endogenous *lin-14* locus and demonstrated that single copy LIN-14 expression is detectible by fluorescence microscopy (Arribere et al., 2014). We could use CRISPR/Cas9-based gene editing to engineer a mutation identical to *lin-14(dp69)* into the *lin-14*::GFP locus. Strains expressing LIN-14::GFP with and without the *lin-14* splice site mutation could be crossed into an *eak-7;akt-1* and *daf-2(e1368)* backgrounds to confirm suppression of the *eak-7;akt-1* Daf-c phenotype by the splice site mutation, and to measure effects of splice site mutation, with or without *lin-14* RNAi on LIN-14 expression.

More broadly, studies of the mechanisms by which heterochronic genes interact with the dauer decision may reveal more about the physiological basis for the dauer decision. Like many developmental decisions, the decision to enter dauer is tightly regulated in developmental time. To enter dauer animals must integrate environmental information at

two critical periods. At the end of the L1 larval stage animals commit to normal development or enter the pre-dauer L2d stage, at the end of L2 they either commit to undergo the dauer diapause or resume reproductive development (Golden & Riddle, 1984). Heterochronic genes may interact with dauer regulatory signaling pathways at each critical period to ensure that the proper developmental program is initiated at the proper time. Interestingly, many heterochronic genes have somewhat functionally conserved roles in regulating developmental timing in other species, suggesting that these studies could reveal broadly conserved mechanisms through which developmental plasticity is coordinated with the proper sequence of developmental events (Moss, 2007).

CEST-1 acts downstream of DAF-16/FoxO to promote longevity

To identify downstream mechanisms through which DAF-16 acts to promote increased lifespan, we profiled the transcriptomes of three strains with DAF-16/FoxO dependent increases in longevity. We then compared the transcriptomes of each long-lived strain with strains in which the increase in lifespan had been fully suppressed by addition of *daf-16*/FoxO mutations which either completely eliminated all gene products, or specifically eliminated *daf-16* transcriptional isoforms that contribute to longevity.

This screen focused our attention on CEST-1, a member of the carboxylesterase family that is both dramatically upregulated by DAF-16/FoxO and required for full lifespan extension in animals treated with *daf-2*/IGFr RNAi. Using a predicted *cest-1* null mutant, we found that CEST-1 was required for full lifespan extension in animals harboring relatively “weak” loss of function mutations in *daf-2*/IGFr but dispensable for lifespan

extension in the context of a "strong" *daf-2*/IGFr mutation. Experiments with CEST-1::GFP expressed under its endogenous promoter confirmed that CEST-1::GFP is robustly induced in *daf-2*/IGFR loss-of-function mutants and suggest that it is expressed specifically in the intestine and localized to the luminal membrane. Overexpression of CEST-1::GFP enhanced the lifespan extension phenotype of a "strong" *daf-2*/IGFr loss of function mutant (*daf-2(e1370)*), and constitutive expression of CEST-1 under an intestine specific promoter modestly extended lifespan in wild-type animals, suggesting that CEST-1 activity is sufficient to promote longevity.

This study has raised several interesting methodological issues which will be important for future attempts to identify DAF-16/FoxO targets, or target genes from other pathways, that promote longevity. To our knowledge this is the first study in which mutants in a DAF-16/FoxO target gene that promotes longevity has been tested for epistatic interactions with multiple long-lived mutants (though a study of this type placed FMO-2 downstream of both the hypoxic response and dietary restriction pathways (Leiser et al., 2015)). The observation that *cest-1* mutations robustly suppress the lifespan extension of some long-lived mutants but not others raises the question of whether different interventions may promote longevity through entirely distinct mechanisms, or alternatively, whether biochemical functions that are required for lifespan extension may be performed by upregulation of partially redundant genes. Given the apparent differences in the magnitude of the reduction in lifespan caused by *cest-1* mutations in the context of *daf-2* RNAi, and different *daf-2* alleles, along with the observation that multiple members of gene classes such as lipases and glutathione-S-transferases show both DAF-16/FoxO-dependent transcriptional regulation and lifespan

effects, we favor the hypothesis that some downstream DAF-16/FoxO targets promote longevity through partially redundant functions. The *C. elegans* genome encodes multiple carboxylesterase enzymes, and, while these enzymes are not transcriptionally upregulated by DAF-16/FoxO in our data, at the young adult timepoint profiled, it will be important to determine whether other carboxylesterases can be upregulated by DAF-16/FoxO, perhaps at other developmental time points, and to generate compound mutants to test the hypothesis that they might act in parallel with CEST-1 to affect longevity. More broadly, it will be important to think critically about the long-lived context that is used to test candidate genes for effects on longevity in future studies. Some long-lived contexts are likely more sensitive than others to lifespan effects, and it will be important to optimize sensitivity and selectivity to identify true regulators of lifespan while screening out false positive effects.

Previous studies have also mostly used RNAi to perform first pass testing of candidate longevity genes. RNAi is prone to false negatives from incomplete knock-down, false positives from off target knockdown, and bacterial strain specific effects. We were able to identify *cest-1* as a novel effector of longevity with candidate testing that used publicly available mutations and test the hypothesis that a predicted carboxylesterase catalytic residue would be critical for longevity using a point mutation that we engineered with CRISPR/Cas9-based gene editing. This suggests that functional testing using mutants is a viable standard for future studies of potential longevity regulators. Importantly, candidate testing using available mutants, most of which were generated by random mutagenesis, also has important caveats. During our candidate testing we observed several strong effects on lifespan that disappeared upon outcrossing, suggesting that it

will be important to control for genetic background in future attempts to identify longevity regulators using mutants. One strategy to minimize false positives might be to identify genes that affect lifespan when mutated using available strains, and then proceed immediately to generating independent mutations using CRISPR/Cas9-based gene-editing with multiple independent guide RNAs, to limit effects stemming from using a previously mutagenized genetic background or from off-target gene editing.

Our data suggest that CEST-1 expression can be sufficient to promote longevity. We do not know what the biological function of CEST-1 is. One strategy to elucidate the biological function of CEST-1 might be to determine whether CEST-1 acts in a pathway with other downstream longevity regulators using genetic epistasis and molecular experiments. We also identified a lipase-like enzyme, *lipl-2*, as a gene that promotes longevity downstream of *daf-2*/IGFr mutations: reports in the literature suggest that lipase activity promotes longevity by generating lipid signals that promote autophagy and downstream transcriptional events (Folick et al., 2015; Lapierre et al., 2011; O'Rourke et al., 2013). Likewise, some carboxylesterase family members have roles in fat metabolism (Redinbo & Potter, 2005). Unlike carboxylesterase and lipase regulators of fat metabolism, which have been characterized to act in lysosomes or in the endoplasmic reticulum, CEST-1::GFP seems to be predominantly localized to the luminal membrane of the intestine, so it is unclear whether it could have similar roles in fat metabolism to enzymes that have been previously characterized. However, it could act in digestion of fatty acids. CEST-1 could also function in digestion of cholesterol esters which could be precursors for cholesterol derived hormones that might regulate longevity, or in digestion of other precursors to beneficial metabolites or signaling molecules. Mammalian

carboxylesterases include enzymes that break down acetylcholine; CEST-1 could function in turnover of an acetylcholine-like signaling molecule. Although it is unclear what the function of acetylcholine might be at the lumen of the gut, neurotransmitter-like small molecules could bind to and affect receptors expressed in the gut to modulate activity of downstream signaling pathways. We could determine whether *cest-1* interacts genetically with lipases or lipase overexpressing strains to regulate lifespan, determine whether it drives autophagy using reporter strains, and determine whether it is involved in similar transcriptional events to lipases using transcriptome experiments in CEST-1 and lipase mutant and overexpressing strains. We could also attempt to identify CEST-1 substrates using unbiased metabolomic approaches. For example we could employ s the 2D NMR methodologies that were used to identify dafachronic acids and looking for metabolites that are altered in a *daf-16* and *cest-1* dependent manner in *daf-2* mutants, using both null and catalytic dead alleles of *cest-1*. Pitfalls of this type of study might be identifying the correct developmental timepoint to profile, since metabolite levels can change on short time-scales and we do not know when CEST-1 activity is required to promote longevity. It may also be difficult and time-consuming to test individual metabolites or biochemically separated groups of metabolites for rescue of lifespan.

It may also be of interest to study in more detail how *cest-1* is transcriptionally regulated. Publicly available data suggests that *cest-1* is transcriptionally upregulated by environmental stresses including ethanol and tunicamycin (Lim et al., 2014; Peltonen et al., 2013). It will be of interest to determine whether *cest-1* mutation is epistatic to other longevity promoting interventions, such as dietary restriction. CEST-1 has not been previously identified as a direct target of DAF-16/FoxO. The CEST-1 genomic region

contains binding regions for other transcription factors including NHR-28 and FOS-1 identified by the modENCODE project using ChIP-SEQ. It may be of interest to determine whether DAF-16/FoxO regulates CEST-1 activity through these transcription factors by crossing mutants into CEST-1::GFP reporter lines or by using gene editing to remove putative transcription factor binding sites (Gerstein et al., 2010; Niu et al., 2011; Schuster et al., 2010). Forward genetic screens for DAF-16/FoxO regulators have largely used dauer arrest as a readout: this is less than ideal since dauer arrest and longevity require DAF-16/FoxO activity in different tissues at different times, and not all genes with effects on dauer arrest will have expected or predictable effects on lifespan. CEST-1::GFP reporters are generally undetectable under a fluorescence dissecting scope in wild-type animals but are robustly induced and easily observable under a fluorescent dissecting scope in *daf-2*/IGFr mutants. Integrated transgenic strains with *cest-1* regulatory elements fused to GFP could enable rapid forward genetic screens for mutations that increase CEST-1 activity; this screen would identify mutations in components of the insulin signaling pathway such as *daf-2* but might also identify novel regulators of DAF-16/FoxO and other longevity pathways. CEST-1::GFP reporters could also be used to identify drugs that might increase lifespan.

Conclusions

This dissertation opened by asking how DAF-16/FoxO activity can delay aging and mortality. Predictably, we do not yet have a mechanistic answer to that question. However, we have both identified a new DAF-16/FoxO target gene with interesting and potentially conserved effects on longevity and validated a strategy for identifying and testing downstream longevity regulating genes that will likely represent a substantial

improvement on strategies that were available previously. We hope that the observations and tools described here will go on to aid in studies which further elucidate the mechanisms of longevity extension, and design interventions to delay or prevent age associated morbidities in humans.

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