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Converging Pathways in the Regulation of Longevity and Metabolism in Caenorhabditis Elegans: A Dissertation

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CONVERGING PATHWAYS IN THE REGULATION OF LONGEVITY AND METABOLISM IN CAENORHABDITIS ELEGANS

A Dissertation Presented

By

SRI DEVI NARASIMHAN

Submitted to the Faculty of the

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November 15, 2010

INTERDISCIPLINARY GRADUATE PROGRAM

CONVERGING PATHWAYS IN THE REGULATION OF LONGEVITY AND METABOLISM IN CAENORHABDITIS ELEGANS

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SRI DEVI NARASIMHAN

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Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
November 15, 2010

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Sitaaron se aage jahaan aur bhii hain
Abhii ishq ke imtihaan aur bhii hain

Beyond the stars, there exists so much more.
The challenges that lie ahead are aplenty.

Taahii zindagii se nahii.n ye fazaayen
Yahaan saikadon kaaravaan aur bhii hain

There is more to this life than mere existence.
And there are many more journeys to undertake.

Kanaa'at na kar aalam-e-rang-o-bu par
Chaman aur bhii, aashiyaan aur bhii hain

Why be content with the colors of the present alone?
Infinite paradises remain to be discovered.

Agar kho gayaa ek nasheman to kyaa Gham
Maqaamaat-e-aah-o-fugaan aur bhii hain

Do not grieve over the loss of your niche.
You will find comfort in new abodes.

Tuu shahiin hai parwaaz hai kaam teraa
Tere saamane aasmaan aur bhii hain

You are a falcon, it is in your nature to soar high above.
Keep flying, the skies are endless.

Isii roz-o-shab me.n ulajh kar na rah jaa
ke tere zamiin-o-makaan aur bhii hain

Do not lose yourself in what is mundane.
For you have new terrains to explore.

Gae din kii tanhaa thaa main anjuman mein
Yahaan ab mere raazadaan aur bhii hain

I once felt solitude amidst the crowd.
Many confidantes surround me here today.

~Allama Iqbal

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Abstract

The lifespan of an organism is determined by a complex array of genetic, environmental and nutritional factors. Yet single gene manipulations have been shown to significantly extend lifespan in several model organisms. Of all the genes that have been studied thus far, components of the insulin/IGF-1 signaling (IIS) pathway have emerged as the most robust regulators of longevity. In addition, IIS also regulates development, energy metabolism and the response to stress in a conserved manner. In *Caenorhabditis elegans*, signaling through this pathway is initiated by activation of the insulin/IGF-1 receptor tyrosine kinase DAF-2, which then activates a PI3-kinase signaling pathway involving additional downstream serine/threonine kinases such as PDK-1, AKT-1, AKT-2 and SGK-1. The concerted action of these kinases results in the negative regulation of the single FOXO transcription factor homolog DAF-16. Under reduced signaling conditions, active DAF-16 is able to translocate into the nucleus and regulate the expression of hundreds of genes regulating longevity, stress resistance, metabolism and development.

The PTEN phosphatase homolog DAF-18, which antagonizes IIS at the level of PI3-kinase, is a major negative regulator of the pathway. However, not much was known about additional phosphatases that negatively regulated the kinases in the pathway. Dephosphorylation is a critical regulatory mechanism by which cellular signaling homeostasis is maintained. Aberrant hyper-activation of growth factor signaling pathways, including IIS, has been implicated in several cancers.

In addition, deregulation of IIS is also closely linked to Type II diabetes. Therefore, the identification phosphatases that balance kinase activity will provide a better understanding of the regulation of the IIS pathway under normal as well as disease conditions. A directed RNAi screen using dauer diapause was conducted in our lab to identify serine/threonine phosphatases that modulated IIS. My work in the Tissenbaum Lab has primarily focused on characterization of the top three candidates from this screen, the genes *pptr-1*, *pdp-1* and *fem-2*. From these studies, we have also uncovered novel crosstalk between the IIS and TGF-β signaling pathways.

In Chapter 2, we demonstrate that PPTR-1, a PP2A phosphatase regulatory subunit negatively regulates the IIS pathway by modulating AKT-1 dephosphorylation. PPTR-1 modulates several outputs of IIS similar to DAF-18. In addition, PPTR-1 co-localizes and physically interacts with its substrate, AKT-1. PPTR-1 modulates dephosphorylation of AKT-1 at a conserved threonine site and we show the molecular conservation of this interaction in mammalian adipocytes. Ultimately, this negative regulation by PPTR-1 results in increased DAF-16 nuclear localization and transcriptional activity.

Next, in Chapter 3, we show how PDP-1 is a novel link between the IIS and TGF-β signaling pathways. Similar to DAF-18 and PPTR-1, PDP-1 regulates multiple outputs of the IIS pathway and promotes DAF-16 activity. Interestingly, PDP-1 acts at the level of DAF-8 and DAF-14, two R-SMAD proteins that function in a TGF-β pathway. Our data suggests that PDP-1 may negatively

regulate TGF-β signaling to downregulate the expression of several insulin(s). Without the insulin ligands, there is less activation of the IIS pathway, and DAF-16 is more active, thereby promoting transcription of genes that act to enhance longevity and stress resistance.

In Chapter 4, we investigate possible crosstalk between IIS and the TGF-β signaling pathways, as the latter was previously considered as a parallel independent pathway. From our studies on PDP-1, we knew that this phosphatase, despite acting in the TGF-β pathway, was a robust modulator of multiple outputs of IIS. Using double mutant combinations as well as RNAi we unravel complex and extensive crosstalk between the two pathways. Importantly, our results suggest that DAF-16 is likely to be the most downstream component of the two pathways.

In Chapter 5, we describe genetic characterization of *fem-2*, and its regulation of the IIS pathway. RNAi of *fem-2* results in robust suppression of dauer formation, similar to *pptr-1* and *pdp-1* RNAi but this phenotype is only observed in the *e1370* allele of *daf-2*. While knockdown of *pptr-1* and *pdp-1* suppress dauer formation of additional alleles of *daf-2*, *fem-2* RNAi has no effect. These results reveal a complex genetic interaction between *fem-2* and the *daf-2* receptor.

Taken together, our results identify several novel regulators of IIS that modulate this pathway by distinct mechanisms.

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List of Abbreviations

IIS: insulin/iGF-1 signaling

ROS: reactive oxygen species

daf: dauer formation abnormal

PTEN: phosphatase and tensin homolog

FOXO: forkhead box class O

TOR: target of rapamycin kinase

TGF-ß: transforming growth factor beta

PP2A: protein phosphatase two (2) A

PPTR-1: protein phosphatase two (2) A regulatory subunit 1

PDP-1: pyruvate dehydrogenase phosphatase homolog 1

PDHc: pyruvate dehydrogenase complex

FEM-2: feminization of XX and XO animals 2

Preface to Chapter 1

The following chapter is an introduction to the biology of aging as well as a

review of the pathways that have been implicated in the regulation of lifespan.

Parts of this chapter are based on the following reviews:

Narasimhan SD*, Yen K* and Tissenbaum HA (2009). Converging pathways in lifespan regulation (Review) *Current Biology* Aug 11;19(15) * Co-first author

Narasimhan SD, Mukhopadhyay A and Tissenbaum HA (2009). InAKTivation of insulin/IGF-1 signaling by dephosphorylation. (Review). *Cell Cycle* Dec;8 (23): 3878-84.

Yen K, **Narasimhan SD** and Tissenbaum HA (2010). DAF-16/FOXO: Many Paths To a Single Fork(head) in The Road (Review). *Antioxidants & Redox Signalling* Aug 1. (Epub ahead of print)

Chapter 1: The Biology of Aging

Part I: Introduction

Living organisms display remarkable diversity in terms of how long they live. Mayflies, upon emergence, survive for no longer than 3 days [1]. In contrast, queen termites live ten to fifteen years [2]. Among vertebrates, the short-lived pygmy goby fish lives a mere 59 days, while giant tortoises more than 150 years old have been recorded [3,4]. Among humans, women outlive men by almost a decade [5]. Despite the relative differences in their length of life, a common eventuality in most living organisms is that with time they will experience an inevitable age-associated decline in function and the ability to survive. Several extrinsic factors such as predation, disease, food availability and temperature can significantly impede the survival rate of an organism and it is likely that in the wild, many animals may never reach old age [4]. Curiously however, eliminating these negative factors still does not prevent the eventual mortality caused by *aging*.

What is aging? Is it a pre-programmed clock that begins ticking as soon as an organism is born? Or is it a progressive decline in the adult years that ensues after the evolutionary function of reproduction has been completed? A biological phenomenon both fascinating and perplexing, aging can be simply defined as the accumulation of changes with time that affects most living organisms [6,7]. These changes include decreased fertility, systemic dysfunction and an increased susceptibility to injury or infection, the majority of which are

undoubtedly deleterious to the organism [8]. In humans, while the phenotypic and physiological changes associated with aging have been recorded by Hippocrates back in 400 B.C, today we have a more detailed understanding of the various tissues and organ systems that are affected. Importantly, dysfunction at these levels manifests in the form of several age-onset diseases such as cancer, type 2 diabetes, atherosclerosis and neurodegenerative disorders (Table 1.1). As a consequence, aging is associated with an increase in mortality over time. Indeed, the rate of death in a population increases as an exponential function of increasing age [4]. This seems to be a near-universal phenomenon, as diverse species exhibit remarkably similar survival curves, again highlighting the fact that with increased age comes the increased probability of death (Figure 1.1).

When and how does this sudden decline in function occur? And if we understand the underlying mechanisms associated with this decline, can we manipulate the system so that we delay the onset of aging? Unfortunately to date, no age-associated biomarker has been conclusively identified. Lipofuscin, for example, is a lipid-enriched pigment that accumulates in different cell types of aged organisms. Lipofuscin is thought to arise from the incomplete lysosomal degradation of damaged mitochondria [9]. It is unclear whether lipofuscin is a marker of organelle dysfunction alone (mitochondria or proteasome) or if its buildup is equally indicative of cellular and even systemic dysfunction. This is again reflective of the fact that the aging is defined as the accumulation of changes at the cellular as well as systemic level. Currently, the most widely used

parameter to measure aging is lifespan. Lifespan, which is defined as the measure of how long an organism survives, correlates well with the aging process. The terms lifespan and longevity are used synonymously in the rest of this study as an indicator of aging in an organism.

Several theories have been posited to explain how and why aging and age-associated decline occurs. Unsurprisingly, these are not mutually exclusive. In the first part of this chapter, I briefly discuss the different theories on the causes of aging and reflect on the available evidence to support these theories. These broadly fall under four categories: the genetic basis of longevity, the effect of mutations, the role of oxidative stress and the relationship between reproduction and aging. The advent of genetic, molecular and genomic tools and the use of model organisms have unraveled novel pathways and mechanisms that modulate longevity. I describe these pathways in greater detail in the second part of the chapter.

Cardiovascular	CNS	Endocrine	Gastrointestinal	Genitourinary and Reproductive	Hematopoetic-Immune	Musculoskeletal	Peripheral Nervous System	Pulmonary	Skin and Subcutaneous Tissue	Special Senses	Miscellaneous
Atherosclerosis, Arteriosclerosis, Ischemic heart disease	Depression	Diabetes mellitus, type 2	Carcinoma of gall bladder	Benign prostatic hyperplasia	Aplastic anemia	Dengerative intervertebral disk disease	Peripheral neuropathy	Chronic interstitial pulmonary fibrosis	Actinic keratosis	Cataracts	Amyloidosis
Calcific aortic stenosis	Glioma, Glioblastoma	Hashimoto thyroiditis	Cholelithiasis	Breast cancer	Autoimmunity	Osteoarthritis Osteomalacia Osteoporosis	Paget bone disease Periodontal disease	Chronic obstructive pulmonary disease	Basal cell carcinoma	Macular degeneration	
Cerebral amyloid angiopathy	Meningioma	Hypo-thyroidism, Myxedema	Colon cancer	Renal carcinoma	Chronic lymphocytic leukemia			Lung cancer	Melanoma	Presbycusis	
Congestive heart failure	Parkinsonism, Parkinson disease	Menopause	Polyps	Nephro-sclerosis	Chronic myelogenous leukemia			Pulmonary embolism	Obesity	Presbyopia	
Giant cell arteritis	Restless leg syndrome		Diverticulitis, Diverticulosis	Prostate cancer	Iron deficiency anemia				Scleroderma		
Hypertension	Shingles, Herpes zoster		Gastric cancer	Urinary bladder cancer	Lymphoma				Seborrheic Dermatitis		
Medial calcinosis	Sleep disorders		Pancreatic cancer	Ovarian cancer	Monoclonal gammopathy, Multiple myeloma	Polymyositis, Dermato-myositis			Squamous cell carcinoma		
Orthostatic hypotension	Subdural hematoma		Atrophic gastritis		Myelo-dysplastic syndrome	Sarcopenia			Squamous cell carcinoma in situ		
Cerebro-vascular disease	Hyperthermia, Hypothermia				Systemic lupus erythematosus				Stasis dermatitis		
Varicose veins	Trigeminal neuralgia				Thymic atrophy				Stasis ulcers		
	Dementia				Pernicious anemia				Xerosis		

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Table 1.1: List of age-onset disorders classified by tissue or organ-system type
[10]
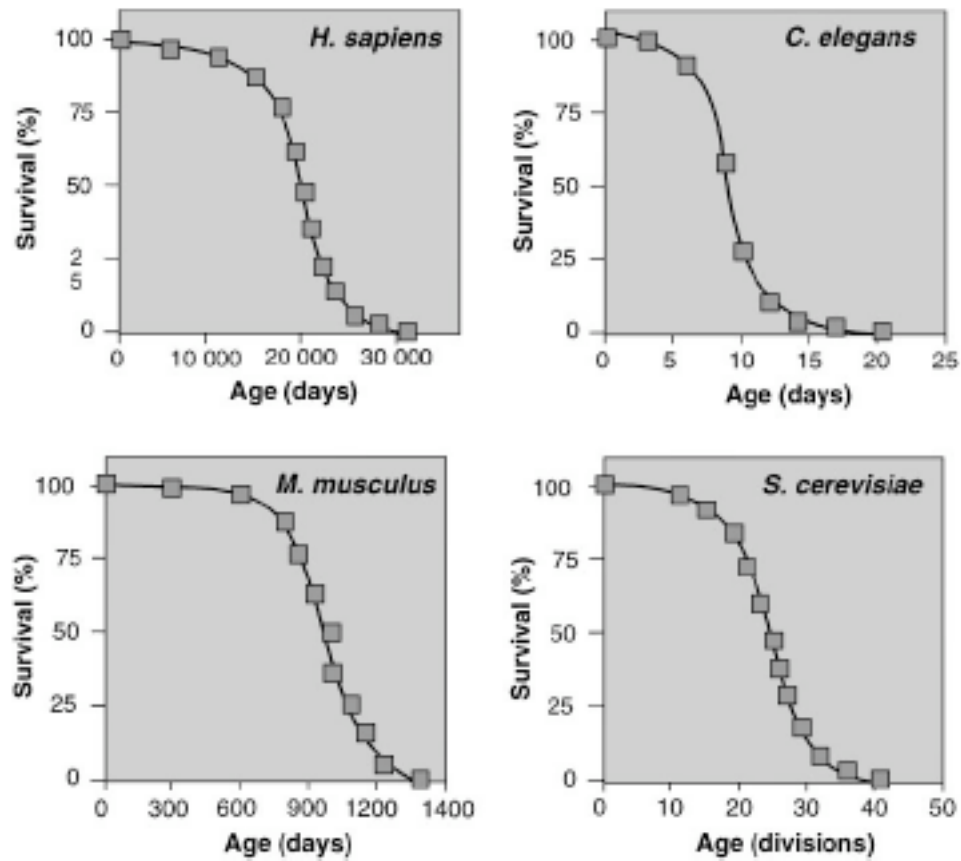
Figure 1.1: Mortality curves for four different species. Despite the differences in their lifespans in terms of absolute number of days, humans, roundworms, mice and yeast, the same shape in their mortality curves [11].

Theories of Aging

<u>A) The Genetic Basis of Aging</u>

Several biological processes such as embryonic development are genetically programmed across phylogeny. Therefore is aging, a seemingly universal phenomenon, also the read-out of a genetic program? Genetics clearly seems to play an important role in regulating how long an organism lives. Among humans, it has been shown that longevity may be an inherited trait, as the children of long-lived parents live longer than those of parents with average lifespans [12] [13] [14].

Single-gene mutations that have been found to extend lifespan in several model systems are also correlated with increased longevity in humans [15,16,17]. Yet if aging were a genetically programmed process, it would be one that reduces reproductive fitness and decreases survival. Such a program would be disadvantageous to an organism in an evolutionary context. In addition, there is a large variability with regards to when age-associated decline affects individuals in a population [8]. This is in sharp contrast to a process such as embryonic development, that is so tightly regulated temporally and spatially Aging, instead, seems to be a consequence of the inability of a system to maintain homeostasis. Several genes that have been identified as important modulators of longevity in model organisms seem to support this in the sense that their actual function is not to enhance or reduce aging *per se* [7,18]. Instead, the modulation of these genes helps the cell survive different biological stresses

that it encounters throughout its lifetime. These stresses may include basic maintenance functions such as repairing mutations and eliminating damaged proteins and organelles or responding to the energy demands of reproduction.

Many of the theories discussed below suggest that aging is ultimately an effect of the accumulation of internal faults within the cell that lead to wear and tear with time. However, external factors such as radiation, pollutants, temperature, pathogens and nutrition can also influence the rate of wear and tear. As discussed later in this chapter, simply cutting the amount of food consumed can significantly delay the aging process. Therefore aging involves a dynamic and complex interplay between the genes of an organism and its environment. The inability to handle these internal and external changes results in the failure to maintain a functional system with time, leading to the onset of age-associated morbidity and mortality.

B) Mutations and DNA Damage

DNA mutations can occur due to cell-intrinsic errors as well as external factors such as mutagens. Mutations can prove to be deleterious to an organism if they are not effectively repaired. There is experimental evidence to suggest that with increasing age, both invertebrates and mammalian models accumulate mutations in their somatic tissues [19]. Deregulation of DNA repair and cell cycle defects have been implicated in several human premature aging or progeroid syndromes such as Hutchinson-Gilford syndrome and Werner syndrome [20].

These diseases are marked by several phenotypic and molecular changes such as changes in nuclear architecture and genomic instability that are in part associated with human aging but affect patients very early in life. It is unclear whether these diseases truly reflect the physiological changes associated with aging, as there are several mutations that can affect basic developmental processes that can also drastically reduce lifespan and lead to general sickness and poor viability.

Telomeres, which protect the ends of the chromosomes, have been the focus of several cellular aging studies. Telomeres get progressively shorter with each cell division and after finite period of divisions, daughter cells arrest from further division and enter cellular senescence. Telomerase, which prevents the loss of telomeres is found in germ cells and stem cells [21]. Mammalian studies have looked at the effect of both telomerase depletion and overexpression on aging phenotypes [22] . While the former results in phenotypes similar to accelerated aging, telomerase overexpression leads to an increased incidence of cancer [22]. In addition, fibroblasts cultured from older people do not reach senescence any faster than those from those who were younger [23]. The relationship between telomeres and aging, therefore remains to be proven.

One of the earliest theories linking mutations to aging was the *Mutation Accumulation Theory* put forth by Medawar in 1952 [4]. This theory suggests that there may be spontaneous deleterious mutations in an organism that only manifest much later in life, after the evolutionary function of reproduction has

occurred. The accumulation of these mutations later in life may be driving the decline associated with aging. An example of this would be humans with Huntington's disease. Most people with this disease live somewhat normal lives for the first three to four decades of their lives despite the presence of mutant *Huntingtin*, and this is the period where reproduction most likely occurs. However, by the fourth decade of life, the rapid and debilitating manifestation of the disease results in systemic decline. While general cognitive decline occurs with age, Huntington's disease only affects a very small percentage of the population. Importantly, since aging is not a genetically programmed process, it is still unclear whether there indeed is a specific activation of deleterious genes during the latter part of life.

The *Antagonistic Pleiotropy Theory* states that genes that have an advantageous function early in life or those that may somehow favor reproductive fitness may prove deleterious to the animal later in life [4,7]. Again, since they do not affect reproduction, they may be selected for despite the negative impact during advanced age. One study in worms supports this idea, as reducing the function of genes that are required for development and growth later in life enhances longevity[24]. In mice, studies suggest that the tumor-suppressor p53 may have antagonistically pleiotropic functions [25],[26]. Hyperactive p53 protects the mice from cancer but causes an accelerated aging phenotype. These studies are somewhat perplexing, since cancer itself is an age-associated disease, and presumably the increased function of a tumor suppressor such as

p53 would be expected to be beneficial to the organism. Further studies may lend support to both of these theories.

C) Oxidative Stress and Aging

The *Free Radical Theory* of aging proposed by Harman in 1956 suggests that reactive oxygen species (ROS) produced as a byproduct of normal metabolism damage macromolecules such as nucleic acids, lipids and proteins by oxidation over time[27]. The increased accumulation of damaged molecules results in aging and its associated disease phenotypes [14],[28] ROS affect cellular components by inducing deletions or modifications in DNA bases, thereby promoting erroneous replication and introducing mutations[29]. Mitochondria are central regulators of cellular energy metabolism and apoptosis. Normal mitochondrial electron transport chain (ETC) function results in the production of small amounts of ROS such as the superoxide anion $O_2.^-$ and hydrogen peroxide (H_2O_2). Increases in cellular energy demands results in increased mitochondrial activity, and as a consequence, increased ROS accumulation over time in different tissues in the body. Mitochondrial DNA (mtDNA) is particularly susceptible to ROS-induced damage, and with increased age, mtDNA replication becomes error-prone, resulting in the accumulation of many mutations [30,31,32]. It is thought that with age, these mutations result in dysfunctional mitochondria that are unable to meet the energy demands of of the cell. One possible consequence of this is the elevated production of ROS, which then gradually damages cellular macromolecules. The role of antioxidant enzymes in

reducing the ROS-induced effects on aging is surprisingly unclear. Studies using superoxide dismutase mimetics have reported conflicting results [33,34]. In addition, while mutations in superoxide dismutase genes reduce resistance to oxidative stress, they do not seem to affect lifespan in general [35]. Importantly, studies suggest that ROS may be important mediators of cellular signaling [29]. Instead of individual types of enzymes eliminating the damage caused by ROS, it is likely that antioxidant defenses work in combination with other molecules such as chaperones in protecting the cell.
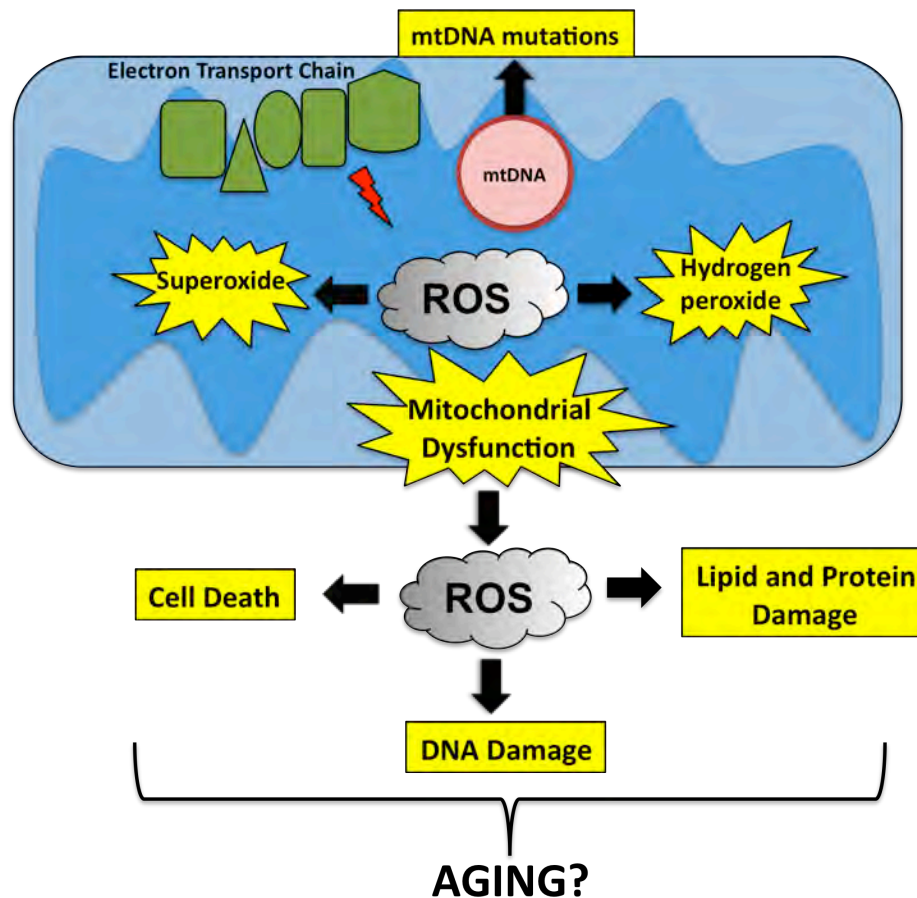
Figure 1.2: Mitochondrial dysfunction and aging. With increasing age, mitochondrial DNA (mtDNA) accumulates mutations and its replication becomes error-prone. As a consequence, mitochondria become dysfunctional and are unable to meet the energy demands of the cell. Small levels of reactive oxygen species (ROS) are usually produced as a byproduct of of normal electron transport chain (ETC) function. Dysfunctional mitochondria lead to elevated ROS production and subsequent damage to cellular macromolecules. (Modified from [18])

D) The Role of Reproduction

Cellular functions such as growth, maintenance and repair of damaged macromolecules require energy. Kirkwood's *Disposable Soma Theory* suggests that the allocation of resources towards maintenance and repair versus reproduction is essentially a trade-off of one versus the other [8]. Reproduction is an energy-consuming process and the resources that could be used towards regular maintenance or repair in the cell are now channeled towards this evolutionarily critical process. In case environmental conditions are unfavorable, reproduction can be delayed and resources are allocated to promote the survival of the organism [36]. This kind of resource allocation has been observed in a wide range of organisms right from worms, fish to higher mammals [4]. The trade-off between reproduction and longevity has been an area of active research, with several interesting observations in different model systems. Germ cells, which are passed on along generations are thought to be immortal, while the rest of the cells that make up the soma are the ones subject to the perils of age-associated decline. Removal of the germline precursor cells in worms results in sterility, but also leads to a 60% increase in lifespan [37]. In addition, male and female fruitflies live longer when maintained separately or when egg laying as well as reproduction is delayed [38,39]. Intriguingly, a study has found that centenarian women were four times more likely to have had children late in their lives as compared to those who survived till their early 70s [40]. The relationship between reproduction and longevity remains an active area of research.
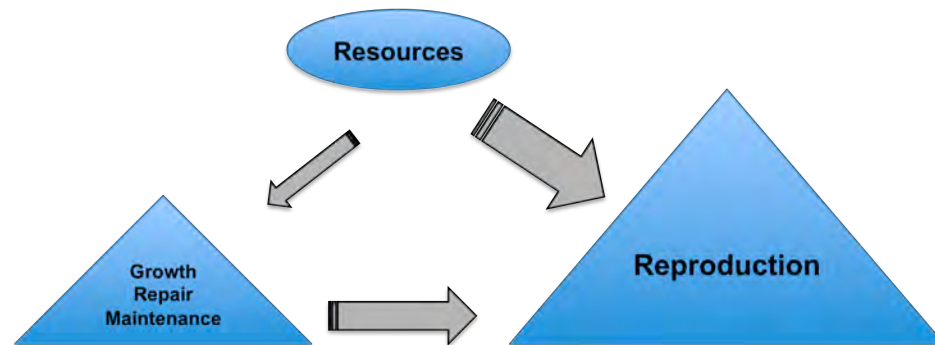
Figure 1.3: Allocation of cellular resources is channeled towards reproduction to maintain evolutionary fitness. Based upon the disposable soma theory, under favorable conditions, an organism will preferrentially ration its energy resources towards reproduction, instead of repairing or maintaining its own system.

C. elegans as a model system for aging research

Testing the different theories of aging is now possible in a laboratory setting because of the development of a number of excellent model systems such as the budding yeast *Saccharomyces cerevisiae,* the roundworm *Caenorhabditis elegans,* the fruitfly *Drosophila melanogaster* and the mouse *Mus musculus. C.elegans*, in particular has been widely used to study the genetics of longevity. *C. elegans* are 1-mm long, free-living organisms that can be propagated in the laboratory by feeding them lawns of *E.coli* on standard agar plates [41] Their transparency allows for ease of observation, especially when using fluorescent reporters to visualize specific tissues (Figure 1.4) [42]. Adult worms contain only 959 cells, and the positions of cells as well as the number of cells is constant, which provides an incredibly rich resource for studying individual cell fate [43]. In addition, *C. elegans* is amenable to genetic manipulations such as RNA interference and with the genome sequence available, powerful forward and reverse genetic tools have been applied to study multiple aspects of cellular function [41,44].

The development of a worm starts as an egg, undergoes embryogenesis and develops through four larval stages, L1-L4 that are separated by molts, before becoming a hermaphroditic adult (Figure 1.4). Single adult worms can produce upto 300 progeny [45]. Worms are constantly sensing their environmental conditions, with growth as well as reproduction only occurring when conditions are favorable. Under unfavorable conditons such as food deprivation and/or

high temperature, increased levels of a constantly secreted pheromone allows worms to enter an alternative stage of developmental diapause known as a dauer [46]. Dauer larvae are resistant to various stresses and hypometabolic – they store elevated levels of fat and metabolize these stores [45]. Early genetic studies identified several mutations that either enhanced or suppressed the ability of the worm to form dauers [47]. These *daf* (dauer-formation abnormal) genes, as I will discuss subsequently, were identified to be part of conserved neuroendocrine signaling pathways that would be later implicated in the regulation of longevity, not just in worms but also in higher organisms.

C. elegans has been extremely useful for aging studies for a number of reasons. They have a short and reproducible lifespan of approximately two weeks, and single gene manipulations have been identified that can significantly increase lifespan by over 100% [48,49]. As previously shown in Figure 1.1, the survival curves of worms and humans share the same shape despite the differences in their lifespans. Despite the evolutionary distance between worms and humans, several hallmarks of aging also seem to be universal. First, worms show an exponential increase in the rate of mortality over time [50]. As mentioned earlier, cellular changes such as alterations in nuclear architecture and increased macromolecular damage have also been observed [51]. Like mammals, aging worms also undergo changes such muscle atrophy, cognitive decline and enhanced susceptibility to infection [51,52]. An additional benefit of working with worms is the ability to identify the role of an individual protein at an

organismal level. As such single-gene manipulations can be directly measured as a phenotypic consequence in a worm using simple well-defined assays such as lifespan assays and oxidative or heat stress assays [50,53,54]. The response to infection can also be assessed by exposing the worms to a pathogen and measuring their survival [52]. Changes in fat storage are qualitatively assessed using the dyes Oil Red O or Sudan Black and quantitatively assessed using gas chromatography, mass spectrophotometry and coherent anti-Stokes Raman spectroscopy (CARS) [55,56,57,58,59]. The molecular conservation of the genes and pathways that regulate longevity in worms has profound therapeutic and economic implications for our understanding of human aging. Humans today are living nearly 35- 40% longer today compared to just 60 years ago, thanks to improvements in healthcare and better nutrition [5]. This has lead to a steady increase in the elderly population worldwide. One of the major challenges with this increase is the treatment of a myriad of age-associated diseases (Table 1.1). As described in the next section, studies from worms and other model systems show that the most robust regulators of longevity are pathways that modulate energy metabolism. Remarkably, polymorphisms in the genes regulating energy metabolism have now been correlated with extreme longevity in humans [16,17].
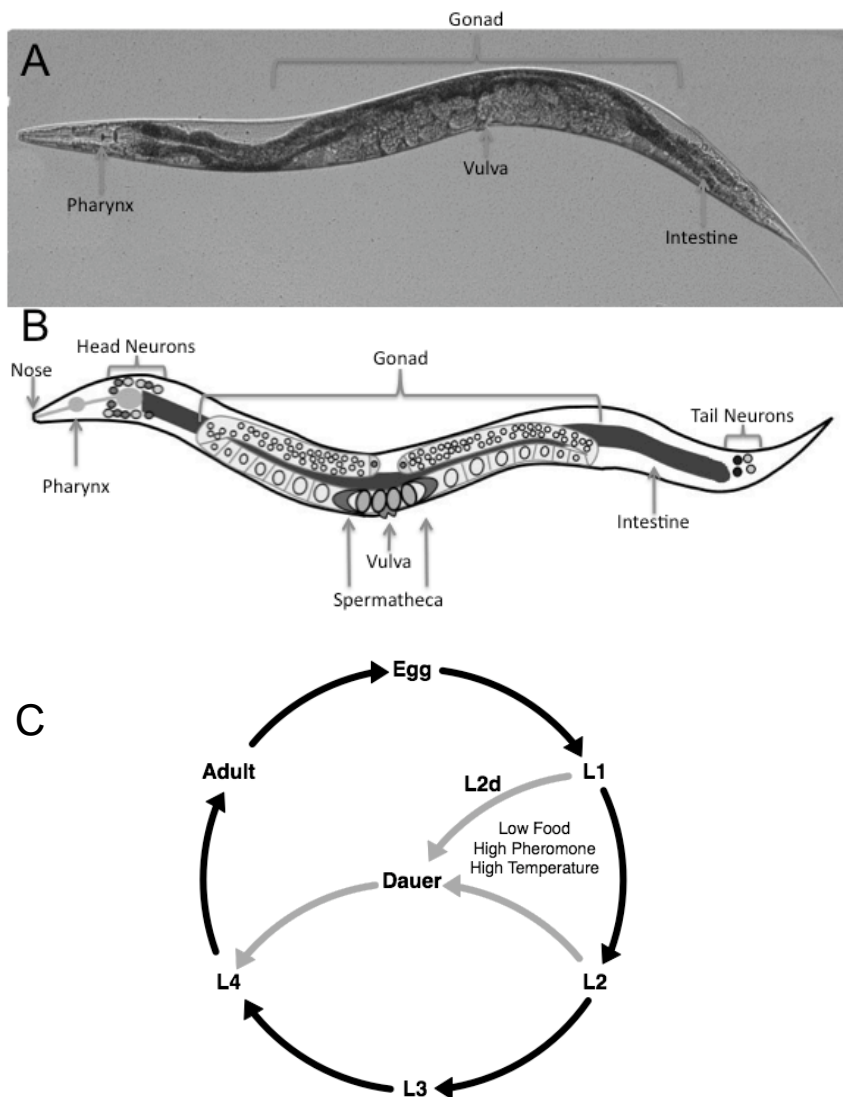
Figure 1.4: A. Differential interference contrast (DIC) image of an adult worm. B. Schematic diagram of the major tissues of the adult hermaphroditic worm. C. Life cycle of *C. elegans*. In a favorable growth environment, the life cycle proceeds from an egg through successive larval stages designated as L1-L4 before becoming an adult. In an unfavorable growth environment, primarily determined by a continuously secreted pheromone along with temperature and food

conditions, worms enter a stage of diapause from the L1 or L2 stage to become stress-resistance dauer larvae.

Part 2: Converging Pathways in the Regulation of Lifespan

Studies in the past two decades have identified more than a 100 genetic determinants of longevity, using approaches such as genome-wide RNA interference (RNAi) and mutagenesis screens, microarrays, and protein arrays [60] [61] [62] [63]. These studies have led to the identification of several important genes that are conserved across phylogeny that are involved in diverse cellular processes, including development, mitochondrial function, energy metabolism, protein translation, and the cell cycle.

The insulin/IGF-1 signaling (IIS) pathway and modulation of food intake by dietary restriction have the most robust effects on lifespan across species. Insulin/IGF-1 signaling is a well-conserved and well-defined pathway that has been shown to regulate longevity in *C. elegans*, *Drosophila*, and in several rodent models [64] (Figure 1.5). In contrast, dietary restriction was long thought of as an extrinsic intervention manifesting in physiological changes that ultimately enhanced lifespan. Dietary restriction, where the actual number of calories ingested by the animal is unknown, differs from calorie restriction, as the exact number of calories consumed is known and the food is also supplemented with extra nutrients such as vitamins and minerals to prevent malnutrition. Recent studies have identified several genes that are necessary for dietary restriction-mediated lifespan extension, suggesting that like insulin/IGF-1 signaling, the longevity-inducing effects of dietary restriction may be regulated by well-defined modulators such as the TOR pathway, olfactory and gustatory signaling, sirtuins

and transcription factors such as SKN-1 and the forkhead protein PHA-4 [65] [66] [67]. Interestingly, the proteins and pathways that are implicated in modulation of dietary restriction have been found to either intersect with or indirectly modulate IIS. Therefore the IIS pathway represents a convergence point for multiple cascades that ultimately modulate longevity and metabolism.

Insulin/IGF-1 Signaling

The importance of the insulin and IGF-1 pathways in regulating energy metabolism and growth has been appreciated for the last sixty years. However clues into the role of these pathways in regulating longevity first emerged in *C. elegans*. Initial studies in worms discovered that single gene mutations resulted in a profound extension in lifespan [60,64]. Further genetic epistasis analysis showed that these genes, *age-1* and *daf-2*, were part of the same genetic pathway [50,60,64,68]. Subsequent cloning studies identified the *daf-2* gene to be equal in homology to both the mammalian insulin and the mammalian IGF-1 receptors and *age-1* as the worm Phosphoinositide (PI) 3-kinase catalytic subunit thus revealing a *C. elegans* insulin/IGF-1 signaling pathway [60,64]. Since then the insulin/IGF-1 signaling pathway has emerged as the best characterized regulator of longevity across species. Indeed, no pathway has been identified with a more pronounced effect on longevity [60,64,69]. Furthermore, the components of insulin/IGF-1 signaling show remarkable molecular and functional conservation from worms to humans (Figure 1.5 and Table 1.2).
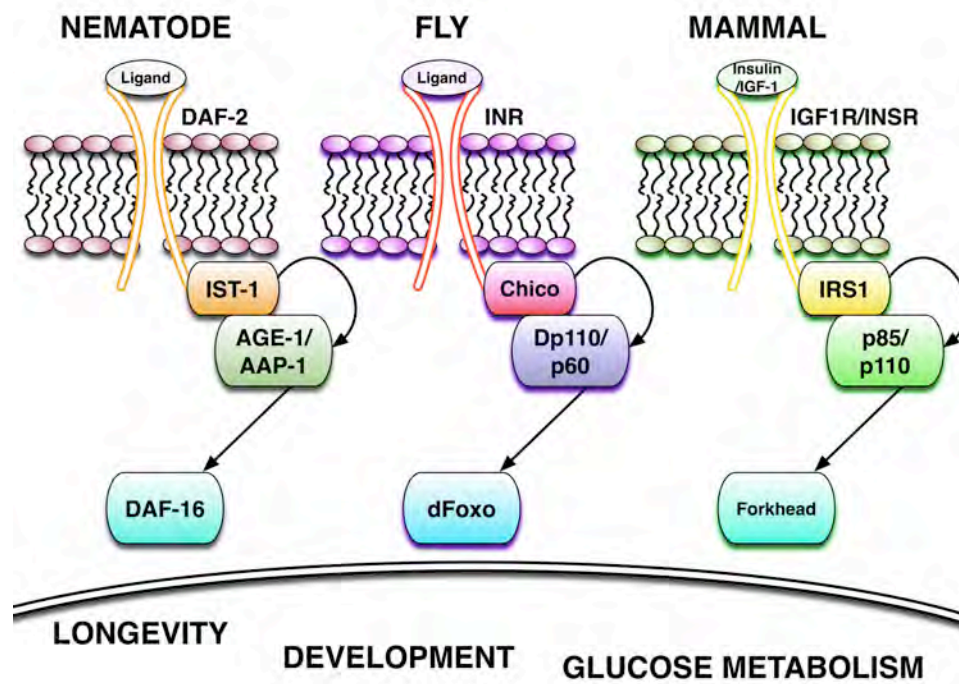
Figure 1.5: Insulin/IGF-1 signaling is conserved across phylogeny. The single receptor in worms and flies is equally homologous to the insulin and IGF-1 receptors in higher organisms. Downstream components such as PI3-kinase and FOXO are conserved in worms, flies and mammals. [75]

A) Insulin-like peptides and the insulin/IGF-1 signaling pathway

At the cellular level, it is well established that changes in blood glucose levels are the main trigger for insulin secretion in mammals. Similarly, in the context of the macroenvironment, organisms constantly sense their surroundings. In *C. elegans,* depending upon the availability of nutrients, several olfactory and chemosensory neurons are thought to regulate the secretion of insulin-like peptides through cyclic GMP and G-protein-coupled receptor signaling pathways [61,69]. Ablation of specific gustatory and olfactory neurons results in increased lifespan, consistent with a role for insulin/IGF-1 signaling in antagonizing longevity [62]. Similarly, studies in flies show that ablation of *Drosophila insulin-like peptide* (*dilp*) neurosecretory cells results in a 10-33% increase in median lifespan [70]. The *C. elegans* genome has an astounding 40 insulin-like (*ins*) genes [71,72](http://www.wormbase.org/ WS198), and *Drosophila* has 7 insulin like peptides (*dilp*) [70]. Why lower organisms appear to have such an expanded family of insulins in comparison to mammals is still not entirely understood. Though the precise ligand that binds to DAF-2 is still unknown, studies on insulins in *C. elegans* revealed that potential ligands may function either as antagonists (*ins-1*), agonists (*ins-7, daf-28*), or both (*ins-18*) [73]. While most studies on insulins in *C. elegans* have been genetic analyses, one study reported biochemical verification of INS-6 binding to the human insulin receptor [74]. Therefore there are still no studies that validate the binding of *C. elegans* insulins to DAF-2. Studies in *Drosophila* have characterized *dilp2, dilp3* and *dilp5* as

important regulators of growth and energy metabolism [70]. Many of these insulins in both worms and flies are expressed in distinct tissues such as the intestine, specific subsets of neurons and in the muscle, and thus in simpler organisms, these ligands may concertedly regulate a neuroendocrine signaling axis modulating development, metabolism, and longevity.

B) Insulin/IGF-1 receptor

Although *C. elegans* and *Drosophila* contain a multitude of potential ligands, only a single receptor that bears homology to the insulin and the IGF-1 receptors has been identified in both organisms: DAF-2 in *C. elegans* and insulin/IGF-1receptor (dInR) in *Drosophila* [70,73]. Reduction of function mutations in *daf-2* result in lifespan extension ranging from 60-100%, indicating that under normal signaling conditions, insulin/IGF-1 signaling promotes growth and development while antagonizing longevity [58,76]. *dInR* homozygous mutant flies are not viable; however, heteroalleleic female flies live up to 85% longer than their wild-type counterparts [64,77]. Downstream of *dInR*, mutations in the fly homolog of the insulin-receptor substrate (IRS) *chico* also extends lifespan up to 48% [70].

In mammals, although the insulin and IGF-1 receptors share high homology, they modulate distinct processes such as metabolism and growth, respectively. Dysregulation of insulin signaling in humans leads to the onset of age-associated debilitating diseases such as type 2 diabetes and cancer. Insulin-receptor knockout mice have a drastically shortened life span due to ketoacidosis [78].

However, studies using tissue-specific insulin-receptor knockout mice reveal a more complex picture. Fat-specific insulin receptor knockout mice not only live almost 20% longer than control littermates, but are also leaner, have increased insulin sensitivity, and express normal IGF-1 levels [78]. Downstream of the receptor, there is contradicting data about whether mice heterozygous for brain-specific IRS2 have an increased lifespan [70]. Several mammalian studies have shown that alterations to the IGF-1-Growth Hormone (GH) axis can increase longevity [64,78]. Mice bearing a mutation in the gene encoding GH and mice with mutation in either *Prop-1* or *Pit-1* (transcription factors involved in pituitary development) show enhanced longevity compared to wild type littermates [64]. These long-lived mice lack several hormones including prolactin and thyroid-stimulating hormone and have diminished levels of GH and neuropeptide Y [78]. Further, GH receptor, GH-receptor-binding protein, and GH-releasing hormone receptor knockout mice show increased lifespan [64]. Interestingly, plasma IGF-1 levels are dramatically low in all of these long-lived mutant mice [79]. In dogs, a polymorphism in IGF-1 is a major determinant of size and a dog's size is inversely correlated with lifespan [80,81]. In humans, a specific polymorphism in the IGF-1 receptor has been associated with increased longevity [16]. Taken together, these data suggest that modifying either insulin receptor or IGF-1 receptor activity can result in changes in longevity across phylogeny.

C) Downstream Kinases

Similar to the signaling pathways in mammals, *C. elegans* has a well-

conserved PI 3-kinase signaling pathway downstream of *daf-2* and *age -1*, the worm homolog of PI 3-kinase catalytic subunit (Figure 1.5 and Table 1.2) [60]. Downstream components such as the phosphoinositide-dependent protein kinase-1 (*pdk-1), akt-1, akt-2,* and serum and glucocorticoid-inducible kinase (*sgk-1)* were identified by both forward and reverse genetic approaches, and mutations in these genes result in lifespan extension (Table 1.2) [60,64]. In the budding yeast *Saccharomyces cerevisiae*, where many aging studies have been performed, there is limited data as to whether a conserved insulin/IGF-1 pathway exists although the conserved Akt homolog *SCH9* is important for regulating lifespan [82]. Remarkably, reduction-of-function mutations in the insulin/IGF-1 signaling pathway not only confer increased longevity but also enhance resistance to heat and/or oxidative stress. Studies in *C. elegans* revealed that these phenotypes ultimately depend upon the single forkhead box O (FOXO) transcription factor *daf-16* [61,62],[69]. Loss-of-function mutations in *daf-16* result in a dramatic suppression of the lifespan extension and stress resistance phenotypes of *daf-2* mutants [61]. The insulin/IGF-1 signaling pathway activates AKT-1/2 and SGK-1 in a PI 3- kinase-dependent manner, and in turn, AKT-1/2 and SGK-1 negatively regulate DAF-16 by phosphorylation [60,62]. Under these conditions, DAF-16 is sequestered in the cytosol by its association with 14-3-3 proteins [8,13]. This regulation is conserved as mammalian AKT and SGK also directly phosphorylate and negatively regulate FOXO proteins [83]. However, under low signaling conditions or in loss-of-function kinase mutants such as *daf-2*

and *age-1,* DAF-16 is less phosphorylated and is able to translocate to the nucleus to transactivate or repress its target genes [60,62,83]

D) DAF-16/FOXO: A central regulator of longevity

What are the important target genes that DAF-16 regulates to confer significant increases in lifespan and stress resistance? A number of approaches, including genome-wide screens, microarrays, and chromatin immunoprecipitation have identified hundreds of genes that are under the control of DAF-16 [60,62,84]. These include molecular chaperones, superoxide dismutases, metabolic genes, and regulators of the cell cycle. It is still unclear how DAF-16 regulates the activity of so many genes, and if all or a subset of these genes actively regulate the phenotypes mentioned above. As a transcription factor, DAF-16 may interact with other co-regulators such as co-activators and repressors to define particular biological processes. Indeed, the nuclear factor SMK-1, the *C. elegans* homolog of SMEK-1, associates with DAF-16 and is required for longevity, innate immunity, and resistance to oxidative stress but not for thermotolerance [60,73]. Several other additional transcription factors and co-activators (HSF-1, HCF-1, CST-1, BAR-1) have been shown to either interact or intersect with DAF-16 in the IIS pathway (Table 1.2). The transcription factor heat-shock factor 1 (HSF-1) is an important regulator of thermal stress in eukaryotes. In response to heat stress, HSF-1 promotes the expression of heat-shock proteins in a DAF-16 dependent manner [62]. Recently, the *C. elegans*

host-cell factor homolog HCF-1 was found to associate with DAF-16 in the nucleus and negatively regulate its transcriptional activity [85]. In addition, CST-1 and BAR-1 have been shown to regulate the transcription of genes involved in oxidative stress in a DAF-16 dependent manner [86]. Their respective mammalian homologs, MST-1 and beta-catenin, also interact with mammalian FOXO, thereby showing the remarkable conservation from nematodes to higher mammals in adaptation to oxidative stress. Thus, depending upon the stressor, DAF-16/FOXO may not only associate with distinct transcriptional cofactors but also regulate the transcription of discrete sets of genes. DAF-16 can be phosphorylated by multiple kinases at residues distinct from the AKT/SGK sites. The AMP-activated protein kinase (AMPK) positively regulates DAF-16/FOXO by phosphorylation [83,87]. Similarly, the c-Jun terminal kinase (JNK) phosphorylates DAF-16 and promotes its nuclear localization, leading to increased lifespan and stress resistance [61,70]. Flies also have a single FOXO homolog (dFOXO) that is phosphorylated in response to signals transduced by dInsR. Overexpression of dFOXO in the adult fat body results in lifespan extension [88]. Similar to worms, a JNK-dependent increase in lifespan and stress resistance depends upon dFOXO, and JNK and dFOXO together can negatively regulate insulin/IGF-1 signaling by repressing the expression of *dilp2* [70]. Much work has focused on DAF-16 and dFOXO in terms of longevity.

In mammals, there are four members of the FOXO family that show overlapping and distinct tissue expression patterns: Foxo1, Foxo3a, Foxo4, and

Foxo6 [34]. Foxo1 plays an important role in angiogenesis and myoblast and adipocyte differentiation, and Foxo1 null mutant mice are embryonic lethal [89,90]. Foxo3a and Foxo4 null mutant mice are viable and grossly similar to their littermate controls but Foxo3a null mutant female mice develop age-dependent infertility [89]. Expression studies reveal that Foxo6 is expressed in the developing brain in embryos as well as the adult brain. Knockout mice have not yet been generated for Foxo6. Although the correlation between FOXOs and longevity has not yet been clearly defined in mammals, these proteins have a well-established role as tumor suppressors [89]. In addition, they regulate the expression of stress-responsive genes such as manganese superoxide dismutase and Gadd45, and may also regulate adult stem cell proliferation [91]. Recent studies have found several FOXO polymorphisms associated with increased longevity across different human populations [15,17].

Pathways that intersect with IIS

Besides the IIS pathway, dietary restriction is a pan-species treatment that can extend an organism's lifespan and health [4] . Recent studies have identified several pathways and proteins that are important for the beneficial effects of DR. Whether the effects of DR occur through IIS or not, is currently not definitive. A number of factors such as genetic studies using non-null alleles or technical differences in the method of DR have lead to inconclusive results. Remarkably, however, the majority of the proteins that have been identified to be important for

modulating the effects of DR are connected to the IIS pathway in a number of ways. Of these, the target of rapamycin (TOR) kinase pathway has been the best characterized as an important regulator of DR. TOR is active in two separate complexes containing both unique and common proteins, and both of these complexes are tightly coupled to IIS. Ribosomal S6 kinase (S6K), a downstream target of TOR complex 1, feeds back into the IIS pathway by phosphorylating and inhibiting IRS-1 [92,93]. Besides phosphorylation by PDK-1 the AKT and SGK kinases of the IIS pathway need to also be phosphorylated by TOR complex 2 to achieve complete activation [94]. Reduction of TOR signaling produces a longevity phenotype and does not further extend the lifespan of IIS mutants [95,96].

The transcription factor SKN-1, an important regulator of oxidative stress and the cellular response to toxins, is directly inhibited by the IIS pathway and also required for DR-mediated lifespan extension [65,97]. The proteins AMPK and SMK-1, which are regulators of DAF-16, have also been found to be important for DR-mediated increases in longevity [98,99]. SMK-1 regulates longevity under conditions of DR through another forkhead protein, the Foxa transcription factor PHA-4[100]. Interestingly DAF-16 and PHA-4 have common consensus binding sites within promoters and the regulation of at least two superoxide dismutases (*sod-1* and *sod-5*) depends upon both of these transcription factors [100]. The sirtuin family of deacetylases has also been implicated in DR and longevity. In *C. elegans,* the lifespan extension mediated by the SIRT1 ortholog *sir-2.1* depends

upon *daf-16*, and SIRT proteins can deacetylate FOXO in mammalian cell culture [101,102]. As described in further detail in Chapter 4, a TGF-β signaling pathway that was previously thought to only modulate dauer diapause has been shown to regulate longevity as well as additional outputs through extensive crosstalk with the IIS pathway [103]. Taken together, these results suggest that instead of a simple linear pathway, IIS is a network composed of multiple inputs and branchpoints that ultimately feed in to modulate lifespan, fat storage and development.

Yeast	Worms	Flies	Mammals
	INS-1-39[1], DAF-28	DILP-1– DILP-7	Insulin and insulin-like growth factor (IGF-1)
	DAF-2 ↑	dINR ↑	Insulin receptor (IR)/insulin-like growth factor-1 receptor (IGF-1R) ↑
	IST-1	CHICO ↑	Insulin-receptor substrate (IRS) ↓
	AGE-1 ↑	Dp110	Phosphoinositide 3-kinase catalytic subunit (p110)
	AAP-1 ↑	p60	Phosphoinositide 3-kinase adaptor subunit (p55)
TEP1	DAF-18 ↓	dPTEN ↓	Phosphatase and tensin homolog (PTEN)
PKH1/PKH2	PDK-1 ↓	Pk61	Phosphoinositide-dependent kinase-1 (PDK-1)
SCH9 ↑	AKT-1/AKT-2 ↑	dAKT	Protein kinase B/AKT
YPK1/YKR2	SGK-1[2] ↑↑	–	Serum and glucocorticoid kinase (SGK)
RTS1	PPTR-1	Widerborst	Protein phosphatase 2A regulatory subunit B56β
FKH1/FKH2/FHL1/HCM1	DAF-16 ↓	dFOXO ↓	Forkhead box O (FOXO) FOXO1, FOXO3a, FOXO4, FOXO6
TOR1 ↑	LET-363 ↓	TOR ↓	Target of rapamycin (TOR)
SCH9 ↑	RSKS-1 ↑	dS6K ↑	Ribosomal S6 kinase (S6K)
KOG1P	DAF-15 ↑	Raptor	RAPTOR (regulatory associated protein of TOR)
AVO3	RICT-1[3]	Rictor	RICTOR (rapamycin-insensitive component of TOR)
HSF	HSF-1	HSF1–4	Heat shock factor (HSF)
KEL1/KEL2/KEL3	HCF-1 ↑	dHCF	Host cell factor (HCF)
SNF1	AAK-2 ↓	SNF1A	AMP-dependent protein kinase (AMPK)
PSY2	SMK-1 ↓	Falafel	Suppressor of Mek-1 (SMEK-1)
SPS1/STE20	CST-1 ↓	Hippo	Mammalian Ste20-like kinase-1 (MST-1)
	BAR-1 ↓	Armadillo	β-catenin
SIR2	SIR-2.1	SIR2	Sirtuin1–7 (SIRT)
HOG1	JNK-1 ↓	Basket	c-Jun N-terminal kinase (JNK)
	SKN-1 ↓	Nrf-2	NF-E2-related factor (Nrf-2)
	–	Keap-1 ↑	Kelch-like ECH-association protein 1 (KEAP-1)
FKH1/FKH2/FHL1/HCM1	PHA-4 ↓	Fkh	Forkhead transciption factor box A (Foxa)
COQ7	CLK-1 ↑	dCLK-1	Clock-1 (mCLK-1) ↑

Arrows indicate the effect of mutations or RNAi on organismal lifespan in wild-type animals: upwards arrow, increased lifespan; downwards arrow, decreased lifespan. [1]Not all ins genes have been characterized: ins-1 mutants are short-lived, ins-7 and daf-28 mutants show an extension in lifespan and ins-18 RNAi/mutants show both phenotypes. [2]sgk-1 lifespan data are currently conflicting. [3]rict-1 mutants show increased lifespan on HB101 bacteria but not OP50 bacteria.

Table 1.2: Modulators of insulin/IGF-1 signaling in yeast, worms, flies and mammals [18]
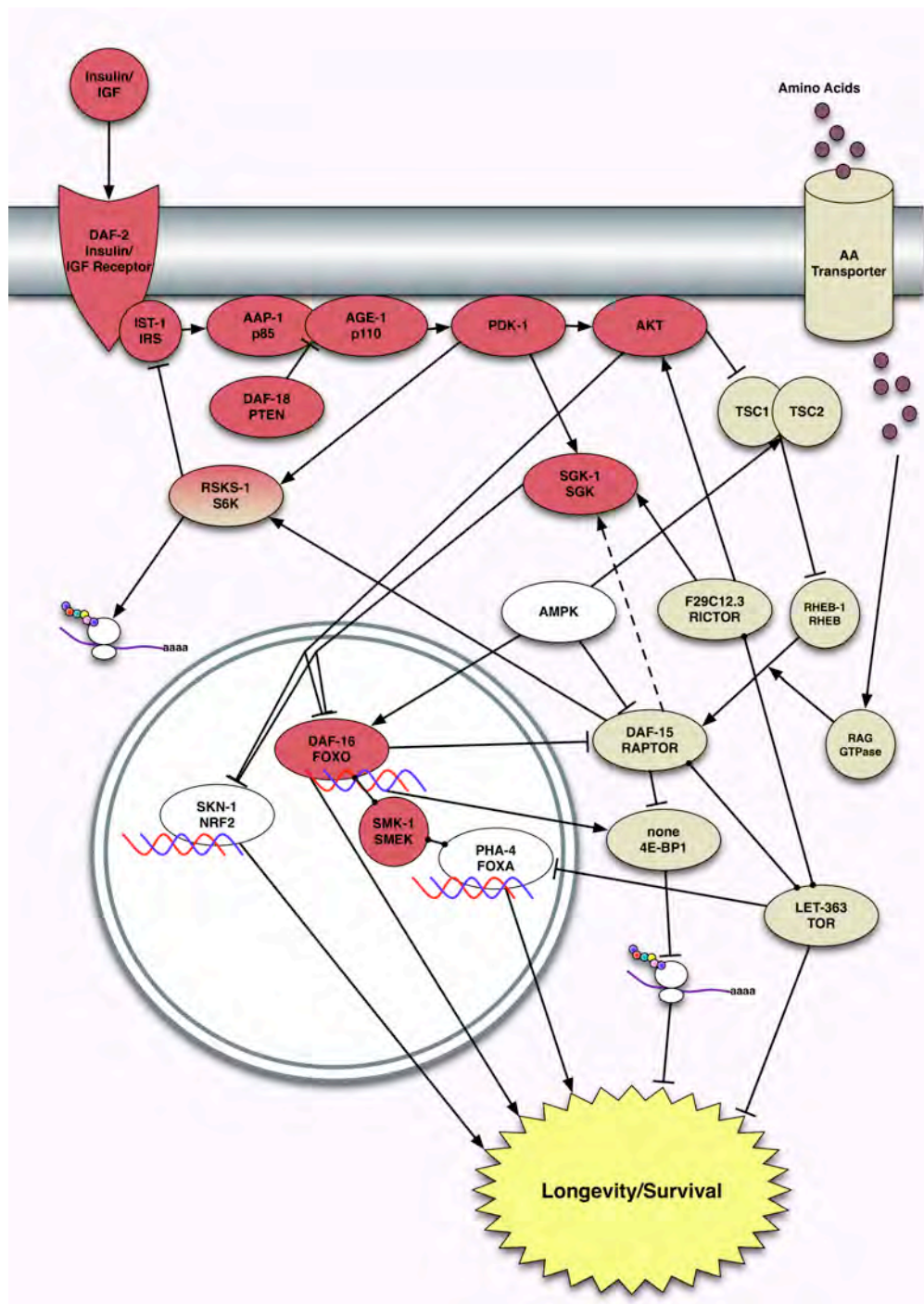
Figure 1.6: The different regulators of longevity converge with the IIS pathway. Modulators of dietary restriction-induced longevity, such as SKN-1, PHA-4, the sirtuins and TOR signaling feed into or intersect with IIS at distinct levels [18].

Part 3: Phosphatases regulating IIS

The different regulators of IIS and DAF-16/FOXO that have been identified until recently include adaptors, chaperones, chromatin modifiers transcriptional co-activators/repressors and a vast number of kinases [18]. Indeed, IIS is a well-studied kinase pathway. However among all of these regulators, protein phosphatases have been surprisingly underrepresented. With regards to the *C. elegans* IIS pathway, the most well studied phosphatase is *daf-18*, the PTEN lipid phosphatase homolog. Mutations in *daf-18* can suppress the long lifespan of *daf-2* mutants and result in increased susceptibility to various stresses [2, 11]. This shows that similar to its mammalian homolog, DAF-18 acts a negative modulator PI 3-kinase signaling and a master regulator of IIS [2]. In contrast, the calcineurin A serine/threonine phosphatase catalytic subunit TAX-6 and its regulatory subunit B CNB-1, are positive regulators of insulin/IGF-1 signaling, as loss-of-function mutants in *tax-6* and *cnb-1* display enhanced longevity [5]. Besides these two families of phosphatases, not much was known about additional protein phosphatases that modulated the kinases in the IIS pathway and DAF-16.

Why is it important to find additional phosphatases that modulate the IIS pathway? Phosphorylation as an important post-translational modification can have several pleiotropic effects on the fate of a protein, including its activation, inactivation or degradation. In particular, in growth factor-induced signal transduction pathways such as the IIS pathway, each phosphorylation step acts as an amplification signal, ultimately regulating diverse processes such as cell

growth, proliferation, energy metabolism and survival [104]. To preserve cellular homeostasis and maintain the balance between aberrant growth and increased apoptosis, it is critical that signals from the kinases are counterbalanced and the phosphorylation events reversed. In this context, protein phosphatases have emerged as central regulators of cellular signaling processes [104].

Protein phosphatases are classified into three main groups: the phosphoprotein phosphatase (PPP) family, the divalent cation (Mg^{2+} or Mn^{2+})-dependent phosphatase (PPM family), both of which dephosphorylate serine/threonine residues, and the protein tyrosine phosphatase family (PTP) [105]. Recent studies have also identified the Asp-based protein phosphatase family, which depend on an aspartate residue for their catalytic activity, as an additional group of serine/threonine phosphatases [106]. The majority of phosphatases encoded in the human genome belong to the PTP family [107,108]. A number of PTPs have been well studied as negative regulators of insulin signaling with PTPα, PTP1B, SHP2 and LAR implicated in dephosphorylation of the insulin receptor as well as the IRS proteins [109,110].

However, the majority of phosphorylation events in the cell occur on serine/threonine residues and yet the human genome encodes for a very small number of phosphatases (including the PPP and PPM families) that dephosphorylate these residues. [106] [111] [112]. Even in the *C. elegans* genome, there are only approximately 60 serine threonine phosphatases in contrast to the 400 plus protein kinases (S. Padmanabhan, Thesis 2009) [113].

How do these few serine/threonine phosphatases effectively counterbalance the activity of multiple kinases and substrates? Once thought of as 'promiscuous' regulators that exhibit little specificity for their targets, some phosphatases have been shown to be capable of dephosphorylating distinct residues even within a single protein [112]. Multiple levels of regulation can determine phosphatase substrate specificity. First, the sub-cellular localization of phosphatases may define a subset of its substrates; the presence of a nuclear, mitochondrial or membrane targeting signal would lead to compartmentalization of the phosphatase and thereby direct it to the local substrate(s) [106] [114] . Secondly, phosphatases may depend on additional co-factors for their activity. For example, PPM phosphatases dephosphorylate their targets without associating with additional structural components but depend upon divalent cations such as Mn^{2+} and Mg^{2+} for their function [115]. Lastly, members of the PPP family such as PP1 and PP2A act as holoenzymes: in addition to the catalytic core that performs the actual dephosphorylation reaction, the enzyme complex often consists of additional structural and/or regulatory subunits that act as a scaffold and determine substrate specificity respectively [112,115].

Reflecting on the part 1 of this chapter, if aging is indeed a consequence of homeostatic imbalance, it becomes all the more necessary to identify and characterize the small pool of serine threonine phosphatases that balance kinase activity in a defined spatio-temporal manner. Given the central and conserved role of IIS in lifespan and energy metabolism regulation, these phosphatases

may provide us with a better understanding of the deregulation of this pathway in

the context of aging as well as several age-onset diseases.

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Preface to Chapter 2

This chapter describes the genetic and molecular characterization of PPTR-1 as a novel negative regulator of the insulin/IGF-1 pathway and modulator of AKT-1 dephosphorylation. The results of this study are further analyzed in a perspective that follows the chapter. The work presented in this chapter was a collaborative effort between Dr. Srivatsan Padmanabhan, a former graduate student and Dr. Arnab Mukhopadhyay, a former postdoctoral fellow and I. PPTR-1 was identified from a RNAi screen previously conducted by S.P. (Padmanabhan, Thesis 2009). S.P. and I both performed all the dauer and heat stress assays, and microscopy to check PPTR-1 localization. A.M. and I did fat assays. A.M. and S.P. did co-immunoprecipitation experiments to show PPTR-1 interacting with and dephosphorylating AKT-1 respectively. I carried out the PPTR-1 overexpression lifespans and growth assays. S.P and A.M. assayed for the role of PPTR-1 on DAF-16 nuclear localization and transcriptional activity. All three of us did the RNAi lifespan experiments. Dr. Greg Tesz from Michael Czech's laboratory did the experiments using 3T3-L1 adipocytes to validate the role of mammalian PPTR-1 (B56) in modulating Akt dephosphorylation. S.P., A.M., Dr. Heidi Tissenbaum and I wrote the manuscript.

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Chapter 2

A PP2A Regulatory Subunit, PPTR-1 regulates

C. elegans Insulin/IGF-1 Signaling by Modulating

AKT-1 Phosphorylation

Summary

The *C. elegans* insulin/IGF-1 signaling (IIS) cascade plays a central role in the regulation of lifespan, dauer diapause, metabolism and stress response. The major regulatory control of IIS is through phosphorylation of its components by serine/threonine-specific protein kinases. In a RNAi screen for serine/threonine protein phosphatases that counter-balance the effect of the kinases in the IIS pathway, we identified *pptr-1*, a B56 regulatory subunit of the PP2A holoenzyme. Modulation of *pptr-1* affects phenotypes associated with the IIS pathway including lifespan, dauer, stress resistance and fat storage. We show that PPTR-1 functions by regulating worm AKT-1 phosphorylation at Thr 350. With striking conservation, mammalian B56ß regulates Akt phosphorylation at Thr 308 in 3T3-L1 adipocytes. In *C. elegans*, this modulation ultimately leads to changes in subcellular localization and transcriptional activity of the forkhead transcription factor DAF-16. This study reveals a conserved role for the B56 regulatory subunit in modulating insulin signaling through AKT dephosphorylation and thereby has widespread implications in cancer and diabetes research.

Introduction

The insulin/IGF-1-like signaling (IIS) pathway is an evolutionarily conserved neuro-endocrine pathway that regulates multiple biological processes including metabolism, development, stress resistance and lifespan [1,2,3,4]. In *C. elegans*, the insulin-like receptor DAF-2 [5] signals through a PI 3-kinase (AGE-1/AAP-1) [6,7] signaling cascade that activates the downstream serine/threonine kinases PDK-1, AKT-1, AKT-2 and SGK-1 [8,9,10]. These kinases in turn function to negatively regulate the forkhead transcription factor (FOXO), DAF-16 [11,12].

Reduction-of-function mutations in serine/threonine kinases upstream of DAF-16 lead to changes in lifespan, development, metabolism and/or stress resistance [1,2,3]. Importantly, loss-of-function mutations in *daf-16* completely suppress these phenotypes [1,2,3,13]. Thus DAF-16 is a major downstream target of the IIS pathway. Phosphorylation of DAF-16 by AKT-1, AKT-2 and SGK-1 results in its nuclear exclusion and sequestration in the cytosol [10,14]. In contrast, under low signaling conditions, active DAF-16 enters the nucleus and transactivates or represses its direct target genes [10,14,15,16,17]. Strikingly, this negative regulation of FOXO/DAF-16 is conserved across species. In mammals, the Akt and SGK kinases can phosphorylate and negatively regulate FOXO [18,19,20] .

Although regulation of the IIS pathway by serine/threonine protein kinases has been extensively studied, little is known about the phosphatases acting in

this pathway. In *C. elegans*, the lipid phosphatase DAF-18 (homologous to mammalian Phosphatase and Tensin Homolog, PTEN), is the only phosphatase that has been identified and characterized as a negative regulator of the IIS pathway [21,22,23,24]. The increased lifespan of *daf-2* mutant worms is suppressed by loss-of-function mutations in *daf-18* or by *daf-18* RNAi [25]. Therefore, to identify additional regulators of the IIS pathway, we performed a directed RNAi screen of serine/threonine protein phosphatases that affect phenotypes regulated by the IIS pathway.

C. elegans development proceeds from an egg, through 4 larval stages into a self-fertilizing, hermaphrodite adult. However, under unfavorable growth conditions such as crowding and low food availability, worms enter a stage of diapause known as as dauer [26]. Upon favorable growth conditions, dauers are able to form reproductive adults. Since worms form dauers constitutively when the function of IIS pathway is reduced by mutations, we took advantage of a temperature-sensitive (ts) allele of *daf-2* for the RNAi screen [27]. We screened for genes that suppressed dauer formation in *daf-2(e1370)* mutants. In this report, we characterize PPTR-1, a regulatory subunit of the PP2A holoenzyme, as an important regulator of development, longevity, metabolism and stress response in *C. elegans*. We show that PPTR-1 acts by modulating AKT-1 phosphorylation and as a consequence controls DAF-16 activity.

Results

1. RNAi screen to identify phosphatases in IIS pathway

To identify the serine/threonine phosphatases in the *C. elegans* genome, we performed *in silico* analyses using both NCBI KOGs (clusters of euKaryotic Orthologous Groups) and WormBase (a *C. elegans* database: http://www.wormbase.org; WS152) annotations. A total of 60 genes were identified for further analysis (Figure 2.1A). We obtained RNAi clones for these phosphatases from the Ahringer RNAi library [28], generated them using available clones from the ORFeome library [29] or cloned them *de-novo* using Gateway Technology (Invitrogen, USA; see Materials and Methods). We were unable to clone 3 of the phosphatase cDNAs and therefore screened a total of 57 candidates (Srivatsan Padmanabhan, Thesis 2009). In addition, we included 6 of the 7 annotated PP2A holoenzyme regulatory subunits (one was not cloned) in the screen for two reasons. First, a preliminary chemical inhibitor screen identified the PP2A family of phosphatases as important regulators of DAF-16 nuclear translocation (Padmanabhan and Tissenbaum, unpublished data). Second, RNAi of the catalytic (C) and structural (A) subunits of PP2A resulted in lethality (data not shown).

daf-2(e1370) carries a mutation in the insulin receptor tyrosine kinase domain that results in a ts phenotype for dauer formation [5]. *daf-2(e1370)* worms arrest as 100% dauers at 25°C whereas at 15°C they have a normal reproductive cycle [26,30]. At an intermediate temperature of 20 ^{o}C, a significant percentage

of *daf-2(e1370)* worms form dauers. Therefore, at this temperature, one can use RNAi to easily assess the contribution of any gene in suppressing *daf-2* dauer formation. For the screen, *daf-2(e1370)* mutants were grown on RNAi-expressing bacteria for two generations, and eggs were picked onto 3 plates for each RNAi clone (Figure 2.1B). The plates were incubated at 20°C and scored 3.5-4 days later for the presence of dauers and non-dauers. Since DAF-18 is the only known phosphatase that negatively regulates the IIS in *C. elegans*, we used *daf-18* RNAi as a positive control in all our experiments. From a total of 63 RNAi clones (57 phosphatases and 6 regulatory subunits), we identified two phosphatases that dramatically decreased *daf-2(e1370)* dauer formation to a level similar to *daf-18* RNAi (Figure 2.1C). Our top candidate, *fem-2* (T19C3.4) regulates *C. elegans* sex determination [31,32]. However, further analysis with an additional *daf-2* allele, *daf-2(e1368)*, revealed that *fem-2* RNAi suppresses dauer formation in an allele-specific manner. *fem-2* RNAi suppressed dauer formation of *daf-2(e1370)* but not *daf-2(e1368)* (Chapter 5) and therefore, we focused on the next top candidate. The next candidate, *pptr-1* (W08G11.4), is a member of the B56 family of genes encoding regulatory subunits of the PP2A protein phosphatase holoenzyme. The *C. elegans* genome contains 7 known PP2A regulatory subunit genes (*pptr-1* and *pptr-2*, B56 family; *sur-6,* B55 family; F47B8.3, C06G1.5, *rsa-1* and T22D1.5, B72 family; currently F47B8.3 is not annotated as a PP2A regulatory subunit according to WormBase Release WS194). To determine the specificity of *pptr-1* in regulating dauer

formation, we re-tested six of seven PP2A regulatory subunits included in the initial screen for their ability to regulate dauer formation in *daf-2(e1370)* worms. As shown in Figure 2.1D, only *pptr-1* RNAi suppressed *daf-2(e1370)* dauer formation comparable to *daf-18* RNAi. We next analyzed the effect of *pptr-1* RNAi on dauer formation of *daf-2(e1368)*. *pptr-1* RNAi significantly suppressed dauer formation of *daf-2(e1368)* (69.2 ± 9.4 % on vector RNAi versus 3.8 ± 4.4% on *pptr-1* RNAi; Table 2.1). Therefore the effect of *pptr-1* RNAi on *daf-2* mutants is not allele-specific and together these results indicate that *pptr-1* may function downstream of *daf-2*. In addition, *pptr-1* is the only PP2A regulatory subunit to affect *daf-2* dauer formation.

A

Phosphatase family	Members	*C. elegans* homologs
PPM	PP2C	10
	PDP	1
PPP	PP1	3
	PP2A	1
	PP2B	1
	PP4	2
	PP5	1
	PP6	1
	PP1 like	40
PP2A regulatory subunits	B55/PR55	1
	B56/PR61	2
	B72/PR72	4

C

Experiment	RNAi	% Dauer ± Std. Dev.	(n)
1	vector	54.4 ± 4.9	285
	daf-18	0.3 ± 0.5	312
	fem-2	0.0 ± 0.0	388
2	vector	17.5 ± 3.7	405
	daf-18	0.0 ± 0.0	395
	pptr-1	1.3 ± 1.6	470

B

daf-2(e1370) grown on RNAi bacteria at 15°C for 2 generations

↓

Pick 100-120 eggs onto three separate plates at 20°C

↓

Score for dauers after 3.5-4 days.

D

Figure 2.1: *pptr*-1, a regulatory subunit of the PP2A holoenzyme, was identified as a top candidate in a directed RNAi screen to identify serine/threonine phosphatases that regulate the IIS pathway.

A) The different families and classes of the phosphatases included in the RNAi screen.

B) A schematic representation of the RNAi screen. All the assays were performed in triplicate.

C) The top two candidates that dramatically suppressed *daf-2(e1370)* dauer formation at 20°C (*fem-2*, and *pptr-1*). Both *fem-2* and *pptr-1* RNAi were able to suppress *daf-2* dauer formation to a similar level as *daf-18* RNAi. Error bars

indicate the standard deviations among the different RNAi plates within one experiment. Data shown [% Dauers ± Std. Dev. (n)] are from one representative experiment.

D) *pptr-1* is the only PP2A regulatory subunit family member that dramatically suppresses *daf-2(1370)* dauer formation. Error bars indicate the standard deviations among the different RNAi plates within one experiment. Data shown are from one representative experiment.

2. *pptr-1* regulates dauer formation through the IIS pathway

To further investigate the role of *pptr-1* in dauer formation, we performed genetic epistasis analysis. In addition to the *C. elegans* IIS pathway, a second parallel TGF-β pathway also regulates dauer formation [33,34]. In this pathway, loss of function mutations in *daf-7* (TGF- β ligand), *daf-1* and *daf-4* (receptors) or *daf-14* and *daf-8* (R-Smads) lead to constitutive dauer formation. Loss-of-function mutations in *daf-3* (Co-Smad) or *daf-5* (Sno/Ski) suppress these phenotypes [35,36,37,38,39]. However, null mutations in *daf-3* do not suppress *daf-2(e1370)* dauer formation [40]. In a *daf-2(e1370);daf-3(mgDf90)* double mutant*,* the input from the TGF- β pathway for dauer formation is essentially removed and presumably dauer formation is regulated by DAF-16. In this strain, the dauer formation was suppressed by *pptr-1* RNAi (94.5 ± 0.8 % dauers on vector RNAi to 42.7 ± 14.6. % dauers on *pptr-1* RNAi; Table 2.1). This data suggests that *pptr-1* controls dauer formation specifically through the IIS pathway and not through TGF-β signaling.

3. *pptr-1* affects longevity, metabolism and stress response downstream of the *daf-2*

In addition to dauer formation, the *C. elegans* IIS pathway also regulates lifespan, fat storage and stress resistance [4,13]. Since *pptr-1* regulates dauer formation specifically via the IIS pathway, we next determined whether this gene could also affect these other important phenotypes.

Mutations in *daf-2* result in lifespan extension [41] that is suppressed by loss-of-function mutations in *daf-18* [22,25]. To investigate whether *pptr-1* can regulate lifespan similar to *daf-18*, we determined whether knocking down *pptr-1* by RNAi could affect *daf-2(e1370)* lifespan. We grew wild type and *daf-2(e1370)* worms on vector, *daf-18* and *pptr-1* RNAi and measured their lifespan (Figure 2.2A). Similar to *daf-18* RNAi, knock down of *pptr-1* resulted in a significant reduction in *daf-2(e1370)* lifespan (mean lifespan of *daf-2(e1370)* on vector RNAi is 33.9 ± 0.7 days , on *pptr-1* RNAi is 27.7 ± 0.9 days and on *daf-18* RNAi is 20.4 ± 0.6 days , p value < 0.0001; Figure 2.2A). In contrast, lifespan of wild type was unaffected by *pptr-1* RNAi (mean lifespan of wild type on vector RNAi is 22.8 ± 0.4 days, is 21.9 ± 0.5 days on *pptr-1* RNAi and 18.6 ± 0.3 days on *daf-18* RNAi; Figure 2.2B). Thus, *pptr-1* affects phenotypes regulated by the IIS pathway, such as lifespan as well as dauer formation Lifespan extension correlates well with increased stress resistance [42 ,43]. For example, *daf-2(e1370)* mutants are not only long-lived but are also extremely resistant to various stresses such as heat and oxidative stress [44,45,46]. Therefore, we next analyzed the effect of *pptr-1* RNAi on the thermotolerance of *daf-2(e1370)* mutants. As anticipated, *pptr-1* RNAi also significantly reduced the thermotolerance of *daf-2(e1370)* mutants (on vector RNAi, *daf-2(e1370)* had a mean survival of 15.2 ± 0.7 hrs, whereas on *pptr-1* RNAi the survival was 13.8 ± 0.5 hrs (p value< 0.006). *pptr-1* RNAi did not affect the thermotolerance of wild type worms; mean thermotolerance was 9.8 ± 0.4 hrs on vector RNAi, versus 9.3 ± 0.3 hrs on *pptr-1* RNAi; Figure 2.2C). In

addition to enhanced lifespan and stress resistance, *daf-2* mutants have increased fat storage [5,47]. We next asked whether *pptr-1* could also affect fat storage in wild type and *daf-2(e1370)* worms using Sudan black staining. Consistent with our lifespan and stress resistance results, *pptr-1* RNAi suppressed the increased fat storage of *daf-2(e1370)* without affecting wild type fat storage (Figure 2.2D). Finally, *daf-2* mutants have a slow growth phenotype [48,49] that is suppressed by knockdown of *daf-16* by RNAi (Figure 2.2E). Similar to *daf-16* RNAi, *pptr-1* RNAi suppresses this slow growth phenotype. Together, these experiments suggest that *pptr-1* regulates multiple phenotypes associated with the IIS pathway in *C. elegans*.
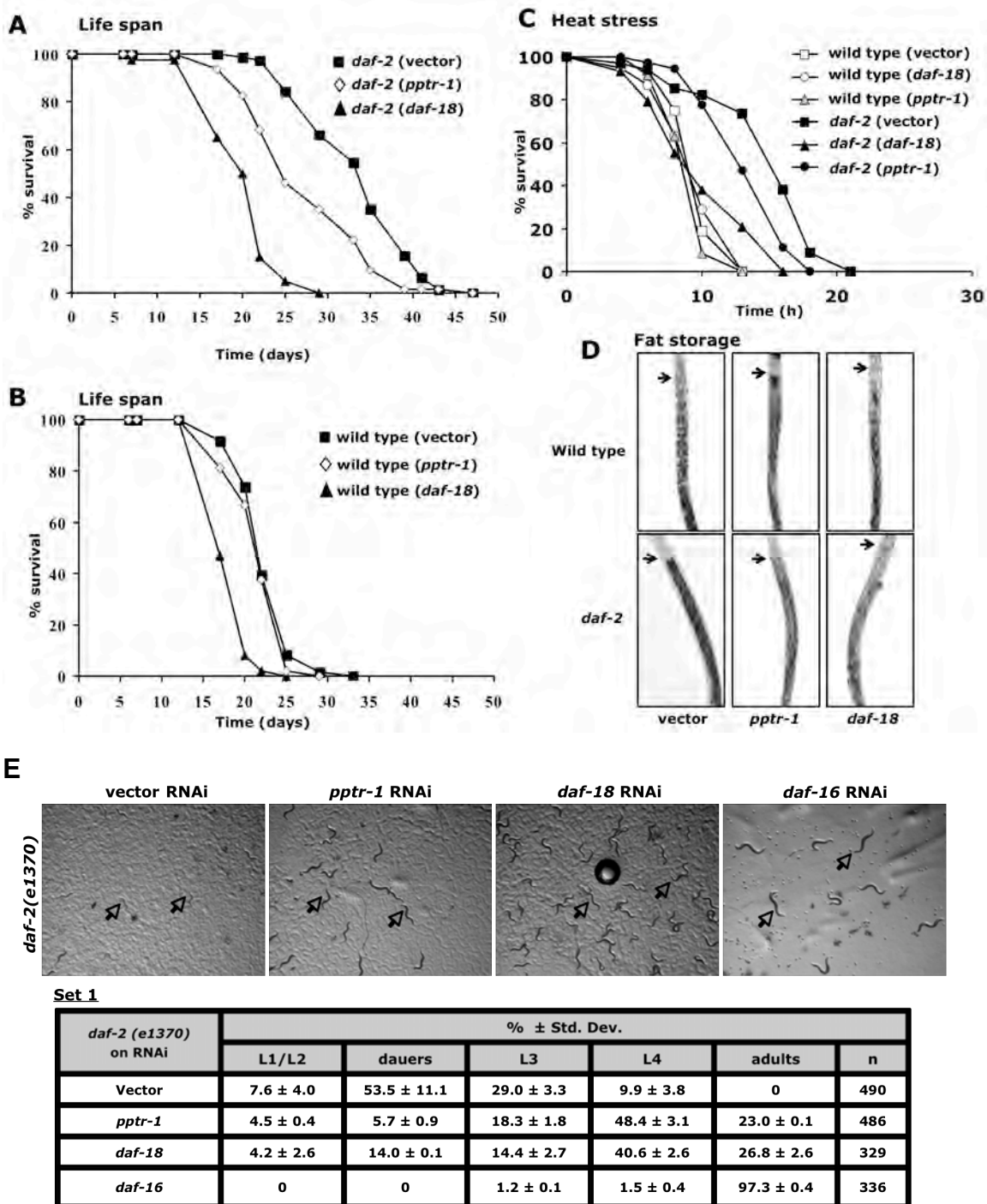
Figure 2.2: *pptr-1* regulates lifespan, thermotolerance, fat storage and growth

through the IIS pathway. Data shown are from one representative experiment.

A) *pptr-1* RNAi significantly reduces the lifespan of *daf-2(e1370)* mutants similar to *daf-18* RNAi (mean life on vector RNAi is 33.9 ± 0.7 days (n=77), *pptr*-1 RNAi is 27.7 ± 0.9 days (n=63) p <0.0001and on *daf-18* RNAi is 20.4 ± 0.6 days (n=40), p <0.0001)

B) *pptr-1* RNAi does not affect the lifespan of wild-type worms (mean lifespan on vector RNAi is 22.8 ± 0.4 days (n=61), *pptr*-1 RNAi is 21.9 ± 0.5 days (n=49). *daf-18* RNAi reduces mean lifespan of wild type worms to 18.6 ± 0.3 days (n=48) p <0.0001).

C) The thermotolerance of *daf-2(e1370)* worms is reduced by *pptr-1* as well as *daf-18* RNAi (mean survival of *daf-2(e1370)* worms at 37 ^{o}C on vector RNAi was 15.2 ± 0.7 hrs (n=34), whereas on *pptr-1* RNAi the survival was 13.8 ± 0.5 hrs (p value< 0.006) (n=36) and 10.3 ± 0.7 hrs (p value< 0.0001) (n=29) on *daf-18* RNAi. *pptr-1* RNAi did not affect the thermotolerance of wild type worms; (mean survival was 9.8 ± 0.4 hrs on vector RNAi (n=32), 9.3 ± 0.3 hrs on *pptr-1* RNAi (n=35) and 9.7 ± 0.4 hrs on *daf-18* RNAi (n=32).

D) Sudan black staining showing that *pptr-1* RNAi reduces the increased fat storage of *daf- 2(e1370)* worms, similar to *daf-18* RNAi but has no effect on wild type fat-storage. Arrows indicate the pharynx. A representative picture from one of three independent experiments (n=30) is shown.

E) *pptr-1* regulates growth in *daf-2(e1370)* mutants, similar to *daf-18* and *daf-16* RNAi.

4. *pptr-1* functions at the level of *akt-1*

Signals from DAF-2 are transduced to the PI 3-kinase AGE-1 to activate the downstream serine/threonine kinase PDK-1 [6,8,25]. PDK-1 in turn phosphorylates and activates three downstream serine/threonine kinases, AKT-1, AKT-2 and SGK-1 [9,10]. These kinases together regulate the transcription factor DAF-16 by direct phosphorylation [10]. Mutations in *daf-16* suppress the enhanced dauer formation of *pdk-1* [8] or *akt-1*/*akt-2* mutants [9,43]. Thus far, our analysis suggests that *pptr-1* functions in the IIS pathway. We sought to identify the potential target of *pptr-1* by performing genetic epistasis experiments.

First we analyzed the effect of *pptr-1* RNAi on dauer formation of *pdk-1* mutant. The dauer formation of *pdk-1(sa680)* was suppressed by *pptr-1* RNAi (95.6 ± 1.0 % dauers on vector RNAi versus 9.5 ± 0.3 % dauers on *pptr-1* RNAi, Table 2.1). In contrast, *daf-18* RNAi had no effect on *pdk-1(sa680)* dauer formation (Table 2.1). Therefore, these results place *pptr-1* downstream of *pdk-1* and are consistent with the current understanding that *daf-18* acts upstream of *pdk-1*.Next, to investigate whether *pptr-1* acts at the level of *akt-1*, *akt-2* or *sgk-1*, we first analyzed dauer formation in *akt-1(ok525)*, *akt-2(ok393)* and *sgk-1(ok538)* single mutants and the *akt-1(ok525);akt-2(ok393)* double mutant. While *akt-1(ok525)*, *akt-2(ok393)* and *sgk-1(ok538)* single mutants do not arrest as dauers at either 20 or 25°C, the *akt-1(ok525);akt-2(ok393)* double mutant forms 100% dauers at all temperatures [43]. To circumvent this problem, we generated double mutants of *daf-2(e1370);akt-1(ok525), daf-2(e1370);akt-2(ok393)* and *daf-*

2(e1370);sgk-1(ok538) and tested these strains for dauer formation on vector, *daf-18* and *pptr-1* RNAi. We reasoned that in a *daf-2* mutant background, the *akt-1, akt-2* and *sgk-1* mutants would exhibit temperature-induced dauer formation. Indeed, all three double mutants were able to form dauers at 20 ^{o}C (Table 2.1 - see panel for vector RNAi). Importantly, *pptr-1* RNAi significantly suppressed dauer formation in *daf-2(e1370);akt-2(ok393)* (36.8 ± 3.8 % dauers on vector RNAi versus 10.8 ± 4.3 % on *pptr-1* RNAi; Table 2.1). In addition, *pptr-1* RNAi suppressed dauer formation of *daf-2(e1370);sgk-1(ok538)* worms (65.4 ± 4.9 % dauers on vector RNAi versus 0 % on *pptr-1* RNAi, Table 2.1). In contrast, *pptr-1* RNAi did not affect dauer formation of *daf-2(e1370);akt-1(ok525)* mutants (vector RNAi is 94.8. ± 3.1 % versus 96.0 ± 1.7 % on *pptr-1* RNAi; Table 2.1). However, *daf-18* RNAi can suppress *daf-2(e1370) akt-1(ok525)* dauer formation (dauer formation was reduced to 10.5 ± 0.8%; Table 2.1). These observations genetically place *pptr-1* at the level or downstream of *akt-1* in the IIS pathway.

Table 2.1: Epistasis analysis of dauer formation IIS pathway mutants

Strains	% Dauer ± Std. Dev. (n)		
	vector RNAi	*daf-18* RNAi	*pptr-1* RNAi
daf-2(e1368)[a]	69.2 ± 9.4 (202)	0 (295)	3.8 ± 4.4 (257)
daf-2(e1370)[b]	17.2 ± 5.9 (517)	0.2 ± 0.3 (397)	9.6 ± 7.3 (460)
daf-2(e1370);daf-3(mgDf90)	94.5 ± 0.8 (589)	40.4 ± 14.0 (308)	42.7 ± 14.6 (329)
pdk-1(sa680)[a]	95.6 ± 1.0 (490)	80.8[c] (52)	9.5 ± 0.3 (180)
daf-2(e1370)	73.0 ± 0.2 (525)	3.5 ± 1.7 (279)	4.1 ± 3.8 (344)
daf-2(e1370);akt-1(ok525)[d]	94.8 ± 3.1 (237)	1.0 ± 0.3 (386)	96.0 ± 1.7 (186)
daf-2(e1370);akt-2(ok393)	36.8 ± 3.8 (336)	5.0 ± 1.1 (610)	10.8 ± 4.3 (583)
daf-2(e1370)	79.1 ± 5.4 (601)	0.7 ± 1.0 (405)	27.2 ± 14.8 (536)
daf-2(e1370);sgk-1(ok538)	65.4 ± 4.9 (338)[e]	0.3 ± 0.4 (303)	0 (364)

All strains were maintained at 15°C and assays were performed at 20°C, unless indicated otherwise. Also, dauer formation of all strains was scored after 3.5-4 days, unless indicated otherwise. Data shown is representative of one experiment.

[a] The experiment was performed at 25 °C
[b] Dauers were scored after 5 days.
[c] In most experiments, the *pdk-1(sa680)* worms failed to hatch on *daf-18* RNAi. This number represents 20% of the eggs picked for this assay.
[d] Dauers were scored after 7-8 days. For *daf-2(e1370);akt-1(ok525)* worms on vector or *pptr-1* RNAi, all the non-dauers were either partial dauers or dauer-like. They did not develop into adults even after 2 weeks.
[e] Dauers were scored after 7-8 days. The *daf-2(e1370);sgk-1(ok538)* strain shows a gro phenotype and worms remain at the L1/L2 stage for 6-7 days at 20°C.

5. PPTR-1 and AKT-1 are expressed in the same tissues

Since *pptr-1* and *akt-1* have a genetic interaction, we wanted to investigate whether they have a common expression pattern. We generated or obtained *akt-1::gfp*, *akt-2::gfp*, *sgk-1::gfp* and *pptr-1::mC-flag* transgenic lines (see Materials and Methods; GFP refers to protein while *gfp* stands for transgene). We made double transgenic worms by crossing *pptr-1::mC-flag* worms to each of the above-mentioned GFP lines. Similar to published data, we observed AKT-1::GFP predominantly in the pharynx, several head neurons, the nerve ring, spermathecae and vulva [9]; AKT-2::GFP in the pharynx (predominantly in the anterior region), somatic muscles, vulva muscles, spermathecae [9]; SGK-1::GFP in amphid neurons, intestine and pharynx (predominantly in the anterior region), somatic muscles, vulva muscles, spermathecae [9]; SGK-1::GFP in amphid neurons, intestine and some tail neurons [10] (Figure 2.3A, B, C middle panel). PPTR-1::mC-FLAG was also observed in the pharynx, head neurons, nerve ring, spermathecae and vulva (Figure 2.3A, B, C left panel). To observe the sub-cellular localization of PPTR-1, we stained *pptr-1::mC-flag* worms with DAPI. We find that PPTR-1 is predominantly cytosolic with little DAPI overlap (Figure 2.3). As shown in Figure 2.3A-C (Merge) there is remarkable overlap between the expression patterns of PPTR-1 and AKT-1. We also observed partial overlap between AKT-2::GFP and PPTR-1::mC-FLAG, predominantly in the pharynx (Figure 2.3B, Merge). SGK-1 and PPTR-1 are expressed in different tissues and we do not see any significant overlap (Figure 2.3C, Merge).
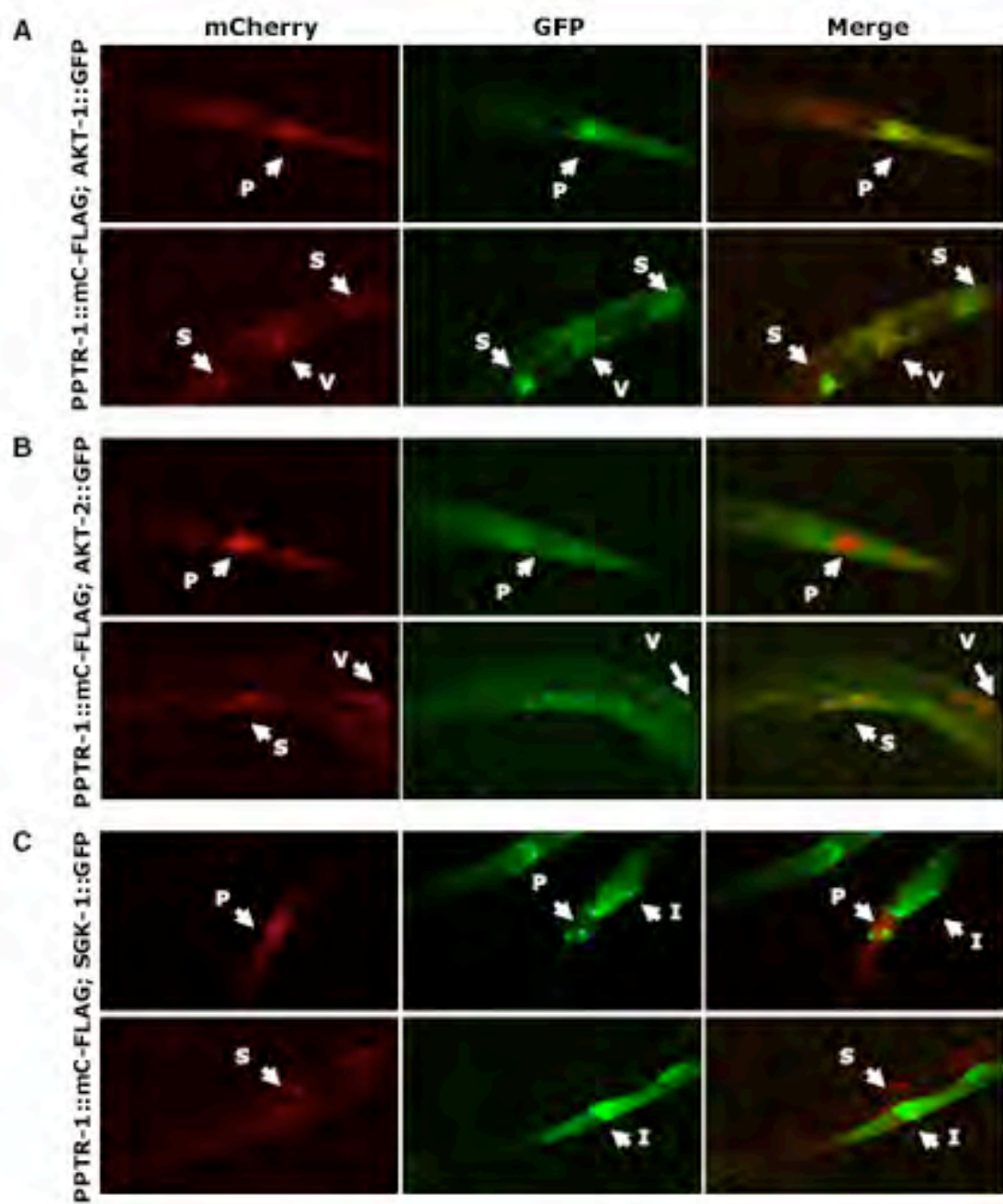
D
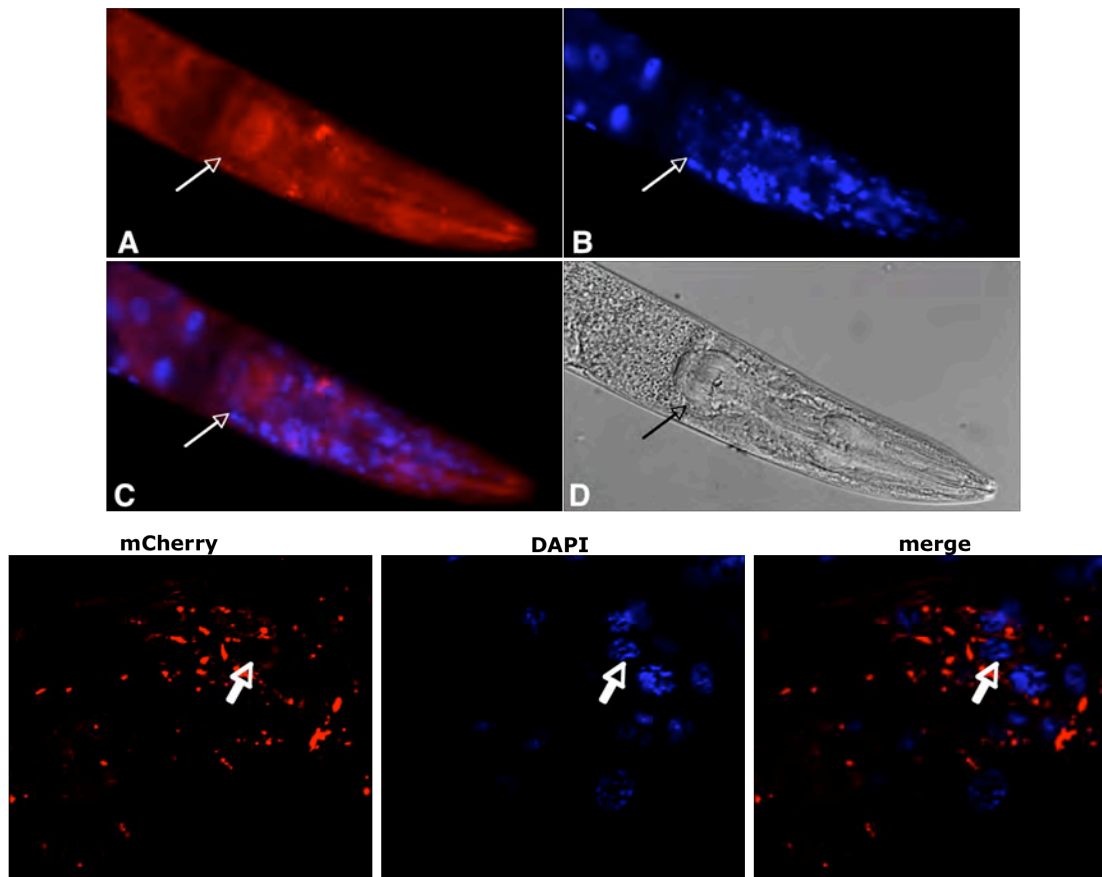
Figure 2.3: PPTR-1 co-localizes with AKT-1. *akt-1::gfp;pptr-1::mC-flag*, *akt-2::gfp;pptr-1::mC-flag* and *sgk-1::gfp;pptr-1::mC-flag* transgenic worms were mounted and visualized by fluorescence microscopy using Rhodamine (mCherry) and FITC (GFP) filters. PPTR-1 expression is observed mainly in the pharynx, vulva and spermatheca (A-C, mCherry).

A) Expression of PPTR-1::mC-FLAG (mCherry) and AKT-1::GFP (GFP) in a *akt-1::gfp; pptr-1::mC-flag* strain. Expression of PPTR-1::mC-FLAG overlaps with AKT-1::GFP (Merge).

B) PPTR-1::mC-FLAG and AKT-2::GFP colocalize in some tissues in a *akt-2::gfp; pptr-1::mC-flag* strain (Merge).

C) SGK-1::GFP and PPTR-1::mC-FLAG do not colocalize in *sgk-1::gfp;pptr-1::mC-flag* transgenic worms (Merge).

Arrows indicate the following tissues: p-pharynx, v-vulva, s-spermatheca, i-intestine

D) Upper panel: High resolution image of *pptr-1::mC-flag* stained with DAPI at 600X magnification. Arrow indicates the pharynx. A) mCherry (B) DAPI (C) Merge (D) DIC.

Lower panel: Subcellular localization of PPTR-1 as visualized in a single plane by spinning disk confocal microscropy after DAPI staining to delineate nuclei. Left, middle and right panels are mCherry at 561nm, DAPI at 405 nm and the merge, respectively.

6. PPTR-1 regulates AKT-1 phosphorylation

Given the genetic epistasis as well as the overlapping expression patterns, we next determined whether PPTR-1 directly interacts with AKT-1 by co-immunoprecipitation (co-IP) in *C. elegans*. For all biochemical experiments, we used the PD4251 strain as a control. This strain contains *Pmyo-3::gfp* with a mitochondrial localization signal and *Pmyo-3::lacZ-gfp* with a nuclear localization signal [50]. This strain will be referred to as *myo-3::gfp*. We prepared lysates from mixed-stage cultures of *akt-1::gfp; pptr-1::mC-flag* and *myo-3::gfp; pptr-1::mcherry-flag* transgenic worms. Following immunoprecipitation with either anti-FLAG or anti-GFP antibody, we found that PPTR-1 specifically interacts with AKT-1 and not with MYO-3::GFP (Figure 2.4A; see Materials and Methods). We also performed co-IP experiments to investigate whether PPTR-1 and AKT-2 interact, since we observed partial overlap in expression pattern of these proteins. We find that PPTR-1 does not interact with AKT-2 (Figure 2.4D). Although we find that PPTR-1::mC-FLAG and SGK-1::GFP interact in our co-IP experiments (Figure 2.4D), our epistasis analyses show no genetic interaction between *pptr-1* and *sgk-1*. Moreover, we observe no overlap in the expression pattern of these two proteins using confocal microscopy (data not shown). Hence, we do not believe this biochemical interaction to have a measurable functional output and did not pursue it further.

In mammals, Akt is activated by PDK phosphorylation at Thr 308 and PDK-2/TORC-2 protein complex at Ser 473 [51,52,53]. In *C. elegans* AKT-1,

these sites correspond to Thr 350 and Ser 517, respectively. We generated affinity-purified phospho-specific antibodies (21st Century BioChemicals, USA; see Materials and Methods) against both sites to further investigate the role of PPTR-1 on AKT-1 phosphorylation. Following immunoprecipitation with anti-GFP antibody from either *akt-1::gfp* or *akt-1::gfp;pptr-1::mcherry::flag* strain, we compared the phosphorylation status at these two sites. We find that overexpressing PPTR-1 can dramatically decrease the phosphorylation of the T350 site while having a marginal effect on the Ser 517 site (Figure 2.4B). As a control experiment, we treated the immunoprecipitated AKT-1::GFP samples with lambda phosphatase and observed loss of the Thr and Ser phosphorylation, showing the specificity of the phospho-AKT antibodies (data not shown). Thus, in *C. elegans*, PPTR-1 functions by directly regulating the dephosphorylation of AKT-1 primarily at the Thr 350 (mammalian Thr 308) site.

7. Mammalian PPTR-1 homolog regulates AKT-1 phosphorylation

Given the evolutionary conservation of the *C. elegans* IIS pathway, we next determined if this mechanism of AKT-1 dephosphorylation mediated by PPTR-1 is also conserved in mammals. The mammalian B56 family of PP2A regulatory subunits has 8 members encoded by 5 genes that express in different tissues [54]. We used 3T3-L1 adipocytes to perform these studies since in this system, there is a well-characterized insulin signaling pathway that is responsive to changes in insulin levels [55,56]. We used microarray data comparing the

expression profiles of fibroblasts to differentiated 3T3-L1 adipocytes [57] to determine which B56 members were expressed in the adipocytes. We identified 2 genes, PPP2R5A (B56a) and PPP2R5B (B56b) as the top candidates. We knocked down either one or both these regulatory subunits by designing Smartpool siRNAs (Dharmacon, USA) and verified the silencing by quantitative RT PCR. Serum-starved siRNA-treated 3T3-L1 adipocytes were then stimulated with increasing concentrations of insulin. The cells were lysed and the proteins analyzed by western blotting using mammalian Akt phospho-specific antibodies (see Materials and Methods). Knockdown of B56b results in a dramatic increase in phosphorylation at the Thr 308 site of Akt with relatively less changes in Ser 473 phosphorylation (Figure 2.4C). However, silencing of B56a had no effect on the phosphorylation status of Akt at either site. We observed that siRNA against at Thr 308 but not at Ser 473. Together, our data suggests that PPTR-1/B56a regulatory subunits function to modulate AKT-1 phosphorylation in a conserved manner across phylogeny.

Figure 2.4: PPTR-1 interacts with and modulates AKT-1 phosphorylation.

A) PPTR-1 directly interacts with AKT-1 in *C. elegans*. AKT-1::GFP and MYO-

3::GFP were immunoprecipitated (IP) using anti-GFP antibody and were analyzed
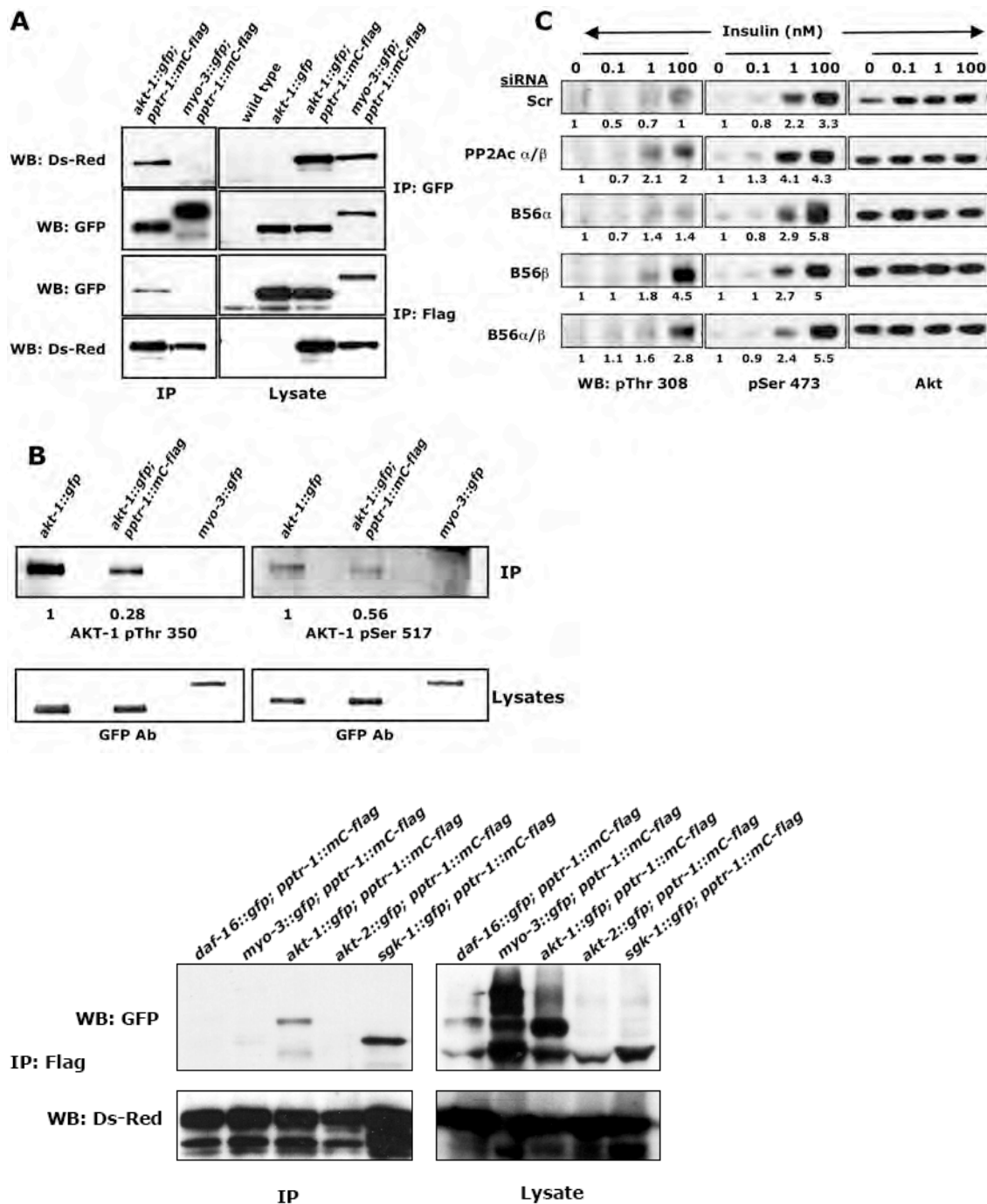
by western blotting (WB) using anti-Ds-Red or anti-GFP antibodies. In addition, PPTR-1::mC-FLAG was immunoprecipitated with anti-FLAG antibody and analysed by WB using using anti-Ds-Red or anti-GFP antibodies. Lysates were used for WB analysis.

B) PPTR-1 overexpression reduces AKT-1 phosphorylation in *C. elegans*. AKT-1::GFP and MYO-3::GFP were immunoprecipitated from *akt-1::gfp, akt-1::gfp;pptr-1::mC-flag* and *myo-3::gfp;pptr-1::mC-flag* followed by western blotting using pThr 350 or pSer 517 antibodies (upper panels). Total lysates were analyzed by western blotting (lower panels).

Quantification of changes in AKT-1::GFP phosphorylation upon PPTR-1 overexpression is shown below each lane.

C) Knock down of the mammalian B56a regulatory subunit by siRNA in 3T3-L1 adipocytes decreases insulin-stimulated AKT phosphorylation at Thr 308. The 3T3-L1 adipocytes were transfected with scrambled (Scr), PP2Aca/b, B56a, B56 b or B56a/b siRNA. These cells were then treated with increasing concentrations of insulin and phosphorylation status of Akt was analyzed by western blotting using pThr 308 (left) and pSer 473 antibodies (middle). Total Akt antibody was used as a loading control (right). Quantification of fold changes in Akt phosphorylation is shown below each lane.

D) PPTR-1 directly interacts with AKT-1 in *C. elegans* but not with DAF-16, AKT-2 or GFP (control). We observe a biochemical interaction between PPTR-1 and SGK-1 in our co-IP experiments but do not see a genetic interaction or any tissue

overlap between these proteins. PPTR-1::mC-FLAG was immunoprecipated (IP) from *akt-1::gfp; pptr-1::mC-flag*, *akt-2::gfp; pptr-1::mC-flag, sgk-1::gfp; pptr-1::mC-flag* , *daf-16::gfp; pptr-1::mC-flag* and *myo-3::gfp; pptr-1::mC-flag* using anti-FLAG antibody and interactions with AKT-1::GFP, AKT-2::GFP, SGK-1::GFP, DAF-16::GFP or MYO-3::GFP (control) and were analyzed by western blotting (WB) using anti-GFP or anti-Ds-Red antibodies.

8. PPTR-1 positively regulates DAF-16 nuclear localization and activity

We next determined the consequences of modulating PPTR-1 dosage on the IIS pathway. In *C. elegans*, one of the major targets of AKT-1 is the forkhead transcription factor, DAF-16. Active signaling through the IIS pathway results in the phosphorylation of DAF-16 by AKT-1, AKT-2 and SGK-1, leading to its nuclear exclusion. However, under low signaling conditions, DAF-16 translocates into the nucleus, where it can directly bind and activate/repress the transcription of target genes involved in dauer formation, lifespan, stress resistance and fat storage [17]. We asked whether *pptr-1* regulates IIS pathway-specific phenotypes by modulating DAF-16 function. Since we observed reduced phosphorylation of AKT-1 upon overexpression of PPTR-1, we first looked at the effect of PPTR-1 overexpression on DAF-16 nuclear localization [14,15,16].

We generated a *daf-16::gfp;pptr-1::mC-flag* strain and then compared the DAF-16 nuclear localization in these worms with a *daf-16*::*gfp* strain (*daf-16::gfp*, a kind gift from the Ruvkun lab, Figure 2.5A). We categorized DAF-16::GFP localization as completely cytosolic, mostly cytosolic, mostly nuclear or completely nuclear. We find that DAF-16::GFP nuclear localization is enhanced when PPTR-1 is overexpressed (Figure 2.5A). To determine the specificity of this response, we used *mCherry* RNAi to effectively knock down *mCherry* expression in *pptr-1::mC-flag* thereby reducing the expression of *pptr-1* transgene (data not shown). Our results show that the enhanced nuclear localization upon PPTR-1 overexpression is suppressed when *pptr-1::mC-*

flag;daf-16::*gfp* worms are grown on *mCherry* RNAi (Figure 2.5A). However, *mCherry* RNAi has little effect on DAF-16 localization in *daf-16*::*gfp* worms. These experiments suggest that increased dosage of *pptr-1* affects DAF-16 nuclear localization. Consistent with its role in the *C. elegans* IIS pathway, we find that overexpression of *pptr-1* significantly increases the lifespan of wild type worms but does not further enhance the lifespan *daf-2(e1370)* worms (Figure 2.5B, mean lifespan of wild type is 23.9 ± 0.3 days, *pptr-1::mC-flag* is 30.1 ± 0.5 days, p<.0001, and the *unc-119(+); unc-119(ed3)* control strain is 22.6 ± 0.3 days).

We next looked at the effect of *pptr-1* RNAi on DAF-16 nuclear localization. For this, we generated a strain with a *daf-2(e1370)*;*daf-16::gfp* strain. At the permissive temperature of 15$^{\mathrm{o}}$C, DAF-16::GFP is excluded from the nucleus in the *daf-2(e1370);daf-16::gfp* worms. However, at the non-permissive temperature of 25$^{\mathrm{o}}$C, progressive nuclear localization of DAF-16::GFP is observed. We grew *daf-2(e1370);daf-16::gfp* worms on either vector, *pptr-1* or *daf-18* RNAi and measured the extent of nuclear/cytosolic localization at 25$^{\mathrm{o}}$C. We find that *pptr*-1 RNAi significantly reduced DAF-16 nuclear localization, similar to the effect of *daf-18* RNAi (Figure 2.5C). Together, these experiments suggest that changes in PPTR-1 levels affect the activity of AKT-1 and as a consequence, modulate DAF-16 sub-cellular localization. Increased dosage of PPTR-1 results in enhancement of DAF-16 nuclear localization while *pptr-1* RNAi causes DAF-16 to be more cytosolic.

9. DAF-16 target genes

DAF-16 regulates the transcription of many downstream genes such as *sod-3*, *hsp-12.6*, *sip-1* and *mtl-1* [17,58,59,60,61]. We next tested the effects of *pptr-1* RNAi on these DAF-16 transcriptional targets. We first tested *sod-3* which has been shown to be a direct target of DAF-16 by chromatin immunoprecipitation [17] and its expression changes in response to modulation of the IIS pathway [61,62]. We grew a *daf-2(e1370);Psod-3::gfp(muIs84)* strain on either vector, *daf-18* or *pptr-1* RNAi to look at the effect on GFP expression. Similar to worms grown on *daf-18* RNAi, *pptr-1* RNAi reduces expression of GFP (Figure 2.5D). Therefore, modulation in the levels of *pptr-1* can affect the expression of direct DAF-16 target genes. We further analyzed the expression of known DAF-16 target genes by quantitative RT-PCR in a *daf-2(e1370)* mutant background. As a control, we analyzed whether each of these target genes expressed in a *daf-16*-dependent manner as previously reported [17,58,59]. As shown in Figure 2.5E,

daf-16 RNAi dramatically suppressed the expression levels of these genes. Next, we tested the effects of either *pptr-1* or *daf-18* RNAi on the expression of these genes. We found that *pptr-1* RNAi also suppressed the expression of these genes to a level similar to *daf-18* RNAi. Taken together, our data suggests that PPTR-1 positively regulates DAF-16 nuclear localization and thereby its activity.

A

Strains	RNAi	Completely Cytoplasmic (%)	Mostly Cytoplasmic (%)	Mostly Nuclear (%)	Completely Nuclear (%)	(n)
pptr-1::mC-flag; daf-16::gfp	vector	34.5	22.9	29.6	12.9	77
	mCherry	79.5	18.6	1.95	0	96
daf-16::gfp	vector	50.0	42.5	5.0	2.5	40
	mCherry	55.3	29.8	14.9	0	47

B

C

Strain	RNAi	Completely Cytoplasmic (%)	Mostly cytoplasmic (%)	Mostly Nuclear (%)	Completely Nuclear (%)	(n)
daf-2(e1370); daf-16::gfp	vector	4.5	40.9	50.0	4.5	22
	daf-18	63.6	45.4	4.5	0	25
	pptr-1	50.0	45.4	4.5	0	22

D

Strain	RNAi	High (%)	Medium (%)	Low (%)	(n)
daf-2(e1370); sod-3::gfp	vector	36.6	46.6	16.6	30
	daf-18	10.0	50.0	40.0	30
	pptr-1	6.6	43.3	50.0	30

E

Strain	RNAi	Transcripts determined by Q-PCR (% of vector RNAi)			
		sod-3	hsp-12.6	sip-1	mtl-1
daf-2(e1370)	daf-16	7.8	10.8	24.7	0.7
	daf-18	46.8	35.2	84.1	17.8
	pptr-1	45.2	52.2	86.7	32.0

F

Figure 2.5: PPTR-1 regulates DAF-16 localization and activity. Data shown are from one representative experiment.

A) Over-expression of PPTR-1 promotes DAF-16 nuclear translocation. On vector RNAi, DAF-16 is more enriched in the nucleus in a *pptr-1::mC-flag;daf-16::gfp* strain, compared to a *daf-16::gfp* strain. This effect is specific to the functional transgene, as knocking down *pptr-1::mC-flag* with *mCherry* RNAi decreases the extent of nuclear DAF-16.

B) Overexpression of PPTR-1 significantly increases the lifespan of wild type worms. Mean lifespan of wild type worms is 23.9 ± 0.3 days (n=154), *pptr-1::mC-flag* is 30.1 ± 0.5 days (n=202), p<.0001, and the *unc-119(+); unc-119(ed3)*

control strain is 22.6 ± 0.3 days (n=145). Data shown are from one representative experiment.

C) In a *daf-2(e1370);daf-16::gfp* strain, DAF-16 is enriched in the nucleus on vector RNAi, whereas on *pptr-1* RNAi as well as *daf-18* RNAi, DAf-16 is mostly cytosolic.

D) *pptr-1* RNAi affects DAF-16 transcriptional activity. *sod-3* is one of the direct targets of DAF-16. *pptr-*1 RNAi reduces *Psod-3::*GFP expression in a *daf-2(e1370);Psod-3*::*gfp(muIs84)* strain, similar to *daf-18* RNAi.

E) Transcript abundance of known DAF-16 target genes decrease when *daf-2(e1370)* worms are grown on *pptr-1* RNAi, similar to *daf-18* RNAi, as detected by real-time PCR.

F) Proposed model illustrating the role of PPTR-1 in the insulin/IGF-1 signaling pathway. Signals from DAF-2 are processed by a PI3-kinase pathway that leads to the phosphorylation and activation of downstream serine/threonine kinases such as PDK-1, AKT-1, AKT-2 and SGK-1. PPTR-1, the PP2A holoenzyme regulatory subunit, regulates the dephosphorylation and activation status of AKT-1 at T350. This in turn affects the nuclear translocation of DAF-16 and the expression of genes involved in lifespan, dauer formation, stress resistance and fat storage.

Discussion

The insulin/IGF-1 (IIS) signaling pathway regulates growth, metabolism and longevity across phylogeny. Given the large number of cellular processes that this pathway controls, understanding the mechanisms that modulate IIS is of paramount importance. IIS is a well-studied kinase cascade but few phosphatases in the pathway are known. Identification of these phosphatases, especially those that counterbalance the activity of the kinases, will provide a better insight into the regulation of this important pathway. *C. elegans* is an excellent system amenable to genetic manipulations including RNAi. In addition, the worm IIS pathway controls several well-defined phenotypes such as lifespan and dauer formation that can be easily quantitated. Therefore, to identify novel phosphatases regulating the IIS pathway, we performed a directed RNAi screen using dauer formation as an output. We specifically looked for serine/threonine phosphatases, as the majority of phosphorylations in the cell, including the insulin signaling pathway, occur on serine or threonine residues [63]. We identified *pptr-1* as a top candidate in our screen. This gene encodes a protein that bears homology to the mammalian B56 family of PP2A regulatory subunits [64]. PP2A itself is a ubiquitously expressed phosphatase that is involved in multiple cellular processes including the regulation of insulin signaling by *C. elegans* PP2A regulatory subunit PPTR-1 modulates insulin signaling by specifically regulating AKT-1 phosphorylation and activity in the context of a whole organism. Furthermore, we show that this mechanism of regulation is

conserved in mammals. We identify PPTR-1 as a novel and integral component of the *C. elegans* IIS pathway.

In our model (Figure 2.5E), PPTR-1 acts to negatively regulate signals transduced through the IIS pathway, ultimately controlling the activity of the FOXO transcription factor DAF-16. Under low signaling conditions, DAF-16 is able to translocate to the nucleus and transactivate or repress its downstream targets. It is well established that AKT modulates DAF-16 sub-cellular localization. Thus, the activity of AKT-1, as governed by its phosphorylation status, directly translates into the activity of DAF-16. In this study, we show that PPTR-1 directly interacts with AKT-1 and regulates its activity by modulating its phosphorylation, predominantly at the Thr 350 site. Less active AKT-1 results in increased DAF-16 nuclear localization. Indeed, DAF-16 is found to be more nuclear throughout the worm when PPTR-1 is overexpressed. As a corollary, knocking down *pptr-1* by RNAi results in less nuclear DAF-16 as well as reduced expression of DAF-16 target genes such as *sod-3*, *hsp-12.6, mtl-1* and *sip-1*. These genes are known to play a combinatorial role in adaptation to various stresses, leading to enhanced dauer formation and increased lifespan. Consistent with the decreased levels of these important genes, *pptr-1* RNAi results in a significant decrease in the dauer formation, lifespan as well as thermotolerance of *daf-2*(*e1370)* worms. In addition, *pptr-1* also regulates other DAF-16-dependent outputs of the IIS pathway such as fat storage. Thus, we find that normal levels of *pptr-1* are important under low insulin signaling conditions.

However, *pptr-1* RNAi does not affect IIS pathway-associated phenotypes in wild type worms. There could be several reasons for this observation. Firstly, under normal signaling conditions, AKT-1, AKT-2 as well as SGK-1 are active and negatively regulate DAF-16. Therefore, changes in the AKT-1 activity alone brought about by *pptr-1* RNAi may not have a significant effect on DAF-16-dependent phenotypes. Secondly, PPTR-1 itself may be negatively regulated by the IIS pathway, leading to increased AKT-1 phosphorylation. Along similar lines, in mammals, insulin signaling can downregulate the expression and activity of the PP2A catalytic subunit [56,65,66]. Thus, under normal conditions, further down regulation of *pptr-1* by RNAi may have no effect. We speculate that in *C elegans*, in response to changing environmental cues, PPTR-1 helps to downregulate the insulin signaling pathway to promote DAF-16 activity, enabling the worm to either enter diapause or enhance its tolerance to stress as adults.

In mammals, Akt controls a myriad of secondary signaling cascades that regulate glucose transport, protein synthesis, genomic stability, cell survival and gene expression [67]. Previous studies have implicated roles for PP2A and PHLPP phosphatases in the negative regulation of Akt [68]. The PP2A inhibitor Okadaic acid can increase Akt phosphorylation predominantly at Thr 308 and enhance glucose transport in adipocytes [69]. Consistent with this, our results show that siRNA knockdown of the PP2A catalytic subunit and more importantly, the B56β regulatory subunit results in enhanced Akt phosphorylation at Thr 308 in 3T3-L1 adipocytes. Thus, our study points at the remarkable functional

conservation of the B56/PPTR-1 regulatory subunit of PP2A in regulating AKT phosphorylation between *C. elegans* and higher mammals. In worms, we also see a modest effect on Ser 517 (equivalent to mammalian Ser 473) phosphorylation by PPTR-1 overexpression. However, we do not observe a difference in Ser 473 phosphorylation in adipocytes. This difference may be explained by the fact that in worms, we are determining the phosphorylation of AKT-1 in the context of a whole organism. Additionally, in mammals phosphorylation state of one Akt site may influence the status of the other [70,71]. We do not see a role for the PP2A B55 subunit (*sur-6)* in the *C. elegans* IIS pathway. However, a recent report using cell culture has implicated the mammalian B55 in the regulation of AKT [68].

Dysregulation of Akt has been implicated in diseases such as cancer and diabetes [69,72,73,74]. In fact, the onset of diabetes is often associated with changes in Akt phosphorylation [74]. In several cancer models, loss of function mutations in the PTEN results in hyper-phosphorylated and activated Akt [75,76,77,78] Our studies show that like PTEN, PPTR-1 acts to negatively regulate the insulin/IGF-1 signaling. Given the important role of PPTR-1/B56 in modulating Akt activity, this protein may be a potential therapeutic target for treatment of diabetes as well as cancer.

Materials and Methods

Strains

All strains were maintained at 15°C using standard *C. elegans* techniques [79]. Double mutants were made using standard genetic methods while transgenic worms were made by microparticle bombardment. For all RNAi assays, the worms were grown for at least two full generations on the RNAi bacteria.

Preparation of RNAi plates

RNAi plates were prepared by supplementing Nematode Growth Media (NGM) media with 100 µg/ml ampicillin and 1 mM IPTG. After pouring, the plates were kept at room temperature (RT) for 5 days to dry. RNAi bacteria were grown overnight at 37°C in LB media supplemented with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline. The next day, the cultures were diluted (1:50) in LB containing 100 µg/ml ampicillin and grown at 37°C until an OD600 of 0.9. The bacterial pellets were resuspended in 1X PBS (phosphate-buffered saline) containing 1mM IPTG. About 200 µl of the bacterial suspension was seeded onto the RNAi plates. The seeded plates were dried at RT for 3 days and stored at 4°C.

Strain Construction

For making double mutants, *daf-2(e1370)* males were mated to either *akt-1(ok525), akt-2(ok393)* and *sgk-1(ok538)* hermaphrodites, respectively. A total of

40 putative F1 cross progeny were singled onto individual plates and allowed to have progeny at 25°C. The F2 dauers were then selected and allowed to recover at 15°C. The recovered dauers were singled and transferred to 25°C and allowed to have progeny. For plates where the F3 progeny formed 100% dauers at 25°C, parents were tested for *akt-1(ok525), akt-2(ok393)* or *sgk-1(ok538)* deletion by PCR.

For making the *daf-2(e1370)*; *daf-3(mgDf90)* double mutant, *daf-2(e1370)* males were crossed to *daf-3(mgDf90)* hermaphrodites. The F1 progeny males (*daf-2(e1370)/+*; *daf-3(mgDf90))* were selected and mated back to *daf-3(mgDf90)* hermaphrodites. Forty putative F2 cross progeny were transferred to individual plates and incubated at 25°C. After 4-5 days, parents were selected from plates where the F3 progeny were 100% dauers and the *daf-3* deficiency was checked by PCR, as described previously[38]. Dauers were recovered to establish the strain.

For co-localization and immunoprecipitation experiments, *pptr-1*::*mC-flag* males were mated to hermaphrodites of each of the following strains *myo-3::gfp* (Fire et al., 1998), *akt- 1::gfp* (SP209) [9]*akt-2::gfp*; *unc-119(+);unc-119(ed3), sgk-1*::*gfp* (BR2773; kind gift from Ralf Baumeister) [10]and *daf-16::gfp* (kind gift from Ruvkun Lab). Potential F1 cross progeny were picked to individual plates and allowed to have progeny. The F2 progeny were examined for progeny homozygous for both GFP and *mCherry* under a fluorescence microscope. For *akt-1::gfp* and *sgk-1::gfp*, the extrachromosomal lines *akt-1::gfp* (SP209) and

sgk-1::*gfp* (BR2777) were integrated into the genome by UV irradiation prior to making the strains. The *daf-2(e1370); daf-16::gfp* and *daf-2(e1370); Psod-3*::*gfp*(*muIs84*) strain were generated by crossing *daf-2(e1370)* males to either *daf-16::gfp* (kind gift from Ruvkun Lab) or *Psod-3*::*gfp*(*muIs84*) hermaphrodites. About 40 putative F1 cross progeny were transferred to individual plates and allowed to have progeny at 25°C. For plates with dauers, the F2 dauers were selected from each plate and allowed to recover at 15°C. The recovered dauers were then checked for the presence of GFP, and GFP-positive worms were transferred to individual plates and incubated at 25°C and allowed to have progeny. Plates where 100% of the progeny were dauers and GFP positive were selected and established as the strain for the assays.

Dauer assays

For the dauer assays, approximately 5 L4 or young adult worms were transferred to the RNAi bacteria and maintained at 15oC. F2 adult worms were then picked to a fresh RNAi plate and allowed to lay eggs. About 120 eggs were picked from these plates onto 3 fresh plates containing the RNAi bacteria and incubated at the indicated temperatures. The plates were scored for the presence of dauers or non-dauers after 3.5-4 days, with some exceptions. For assays involving *daf-2(e1370); sgk-1(ok538)*, the strain is slow growing with a prolonged L1/L2 arrest and only forms dauers on vector RNAi after 7-8 days. Similarly, *daf-2(e1370); akt-1(ok525)* worms grown on vector and *pptr-1* RNAi

were also scored after 7-8 days. The *pdk-1(sa680)* worms have an Egl phenotype. For the *pdk-1(sa680)* dauer assays, eggs were obtained by hypochlorite treatment of gravid adults worms grown on vector, *daf-18* and *pptr*-1 RNAi plates [79].

Lifespan assays

All lifespan analyses were performed at 15ºC. Strains were synchronized by pickingeggs on to fresh RNAi or OP50 plates and allowed to grow for several days until they became young adults. Approximately 60 young adult worms were transferred to each of 3 RNAi plates for every RNAi clone tested (vector, *daf-18* and *pptr-1).* For lifespan experiments with overexpression strains, approximately 60 young adult worms were transferred to 3 fresh OP50 plates for every strain tested. Lifespans were performed on RNAi plates or OP50 plates overlaid with 5-fluorodeoxyuridine (FUDR) to a final concentration of 0.1 mg/ml of agar [80]. We observed significantly fewer worms bursting at 15ºC by transferring young adult animals rather than L4 animals to FUDR plates. Worms were then scored as dead or alive by tapping them with a platinum wire every 2-3 days. Worms that died from vulval bursting were censored. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

daf-2(e1370) Growth Assay

daf-2(e1370) worms were maintained on vector, *pptr-1*, *daf-18* and *daf-16*

RNAi plates for two generations at 15°C. Approximately 100-150 eggs were picked on to two fresh plates for every RNAi clone tested and the plates were incubated at 20°C. Worms were scored based upon their stages as larval stages 1/2 (L1/L2), dauers, larval stage 3 (L3), larval stage 4 (L4) or adults after 3.5 days.

Heat stress assays

Wild type and *daf-2(e1370)* animals were maintained on RNAi bacteria at 15°C. From these plates, approximately 30 young adult worms were picked onto fresh vector, *daf-18* and *pptr-1* RNAi plates. These plates were shifted to 20°C for 6 hrs. The plates were then transferred to 37oC and heat stress-induced mortality was determined every few hours until all the animals were dead. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

Fat staining

Sudan Black staining of stored fat was performed as previously described (Kimura et al., 1997). Briefly, wild type and *daf-2(e1370)* worms on RNAi plates were synchronized by picking eggs on to fresh RNAi plates and grown until the L3 stage. The worms were then washed off the 3 plates and incubated in M9 buffer for 30 minutes on a shaker at RT. After 3 washes with M9 buffer, the worms were fixed in 1% paraformaldehyde. The worms were then sequentially

dehydrated by washes in 25%, 50% and 70% ethanol. Saturated Sudan Black (Sigma, USA) solution was prepared fresh in 70% ethanol. The fixed worms were incubated overnight in 250µl of Sudan Black solution, on a shaker at RT, mounted on slides and visualized using the Zeiss Axioscope 2+ microscope.

DAF-16::GFP localization assay

The *daf-2(e1370);daf-16::gfp* strain was maintained at 15_oC on vector, *daf-18* or *pptr-1* RNAi plates. About 20-25 L4 or young adults were transferred to fresh RNAi bacteria and the plates were shifted to 25_oC for 1hr. The worms were then visualized using Zeiss Axioscope 2+ microscope. Worms were classified into four categories based on the extent of DAF-16::GFP nuclear-cytoplasmic distribution. +: completely cytoplasmic; ++: nuclear in some tissues but cytoplasmic in majority of the tissues; +++: cytoplasmic in some tissues but nuclear in majority of the tissues; ++++: nuclear localization in all tissues [10]

Psod-3::GFP expression

daf-2(e1370); Psod-3::*gfp*(*muIs84*) worms were grown at 15_oC on RNAi plates as described above. About 25-30 L4/young adults were transferred to fresh RNAi plates and shifted to 25_oC for 2 hrs. The expression of GFP was visualized using Zeiss Axioscope 2+ microscope. Worms were classified into three categories based on the intensity of GFP expression. High: bright GFP expression seen throughout the worm; Medium: Low GFP expression in the

worm body; Low: weak or barely detectable GFP expression in the body. GFP expression in the head region does not change dramatically.

Transgenic worms

A 3 kb sequence of the *pptr-1* promoter and the *pptr-1* ORF were cloned into separate entry vectors [29,81] using Gateway Technology (Invitrogen, USA) and confirmed by DNA sequencing. The promoter and ORF were then combined using multi-site Gateway cloning into the *pSCFTdest* vector to create the *pSCFT-pptr-1*. An *unc-119* promoter::ORF fusion mini-gene was constructed as described [82] and cloned into *pUC-19* vector between *EcoR*I sites. The *unc-119* mini-gene insert was then excised by *EcoR*I digestion, followed by gel purification, and then blunt ended with T4 DNA Polymerase (Roche Biochemicals, USA) and cloned into *pSCFT-pptr- 1* (at the filled-in *Sph*I site) giving rise to the *pSCFT-pptr-1-unc-119* vector. This construct was used in biolistic transformation (Biorad, USA) of *unc-119(ed3)* mutants [82,83] Integrated lines were back-crossed four times to wild-type and used for further analysis. For the *akt-2::gfp* construct, a 3 kb sequence of the *akt-2* promoter was cloned into the corresponding entry vector and the *akt-2* ORF from the ORFeome were combined using multisite Gateway technology into the R4-R2 destination vector [29]to create *akt-2::gfp-unc-119(+)* vector. This vector was verified by restriction digestion and integrated transgenic lines were obtained by biolistic transformation (Biorad, USA) [82,83]

C. elegans immunoprecipitation (IP) and western blotting

Transgenic worms were grown in three 100 mm plates seeded with OP50 bacteria at 20 ^oC. Worms were harvested by washing with M9 buffer and pellet collected by centrifugation. The pellet was resuspended in 250 µl lysis buffer (20 mM Tris-Cl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 25 mM β - glycerophosphate, Protease inhibitor cocktail (Roche Biochemicals, USA), pH 7.4). The worms were sonicated with Bioruptor (Diagenode, USA) using maximum power output (1 min sonication, 2 min off-repeated 10 times). The lysate was cleared by centrifugation and protein content estimated by Bradford method. Lysate equivalent to 1.5 mg total protein was pre-cleared with 50 µl of protein-G agarose beads, fast flow (Upstate, USA) and then immunoprecipitated overnight at 4 ^oC using either anti-GFP monoclonal antibody (Sigma, USA) or anti-FLAG M2 gel (Sigma, USA). The following morning, 50 µl protein-G agarose beads, fast flow were added to the GFP IP to capture the immune complex. The agarose beads were then washed 5 times with lysis buffer. Following this step, the beads were boiled in Laemelli's buffer. For western blot analysis, immunoprecipitated protein samples was resolved on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST (Tris Buffered Saline containing 0.05% Tween 20, ph 7.4) containing 5% non-fat milk at RT for 1 hour. Membranes were then washed with TBST and incubated overnight with 1:1000 dilutions of antibodies in TBST containing 5% non-fat milk 4 ^oC. Membranes were washed 3 times with TBST and then incubated with

TBST containing 5% non-fat milk containing a 1:10,000 dilution of the secondary antibody.

Antibodies used for western were:

Living Color DsRed antibody (Clontech, USA; Catalog no. 632496)

Living Color Rabbit polyclonal GFP antibody (BD Biosciences, USA; Catalog no. 632460)

Monoclonal mAb 3e6 GFP antibody (Invitrogen, USA; Catalog no. A11120)

Anti-FLAG M2 Affinity Gel (Sigma, USA; Catalog no. A2220).

C. elegans phospho-AKT western blotting:

Transgenic worms were grown at 20 ^{o}C in 3-4 large (100 mm) plates seeded with OP50. Worms were collected by washing with 1 X PBS and the pellet was then immediately frozen in dry ice. Around 500 µl lysis buffer, supplemented by Sigma Phosphatase inhibitor cocktails I and II (50x) and Protease inhibitor cocktail (Roche Biochemicals, USA) was added to the pellet and sonicated using a Misonix (3000) sonicator (Misonix, USA; power output set at 4, 3 pulses of 10 secs each with 1 min interval between pulses). The lysates were clarified by centrifugation at 13000 rpm for 10 mins at 4°C and the protein content estimated by Quick Bradford (Pierce). About 3.5 µg of anti-GFP monoclonal antibody (3E6, Invitrogen USA) was used for each IP from lysates containing 1.7 mg protein in a volume of 1ml. IPs were performed overnight at 4°C and antibody-protein complexes were captured using 50 µl of protein-G

agarose beads, fast flow (Upstate, USA) for 2 hrs at 4°C. The pellets were washed 3 times with lysis buffer supplemented by protease and phosphatase inhibitors and boiled in Laemelli's buffer. The IP samples were then resolved on a 10% SDS-PAGE, western blotted and analyzed with phospho-specific antibodies.

Mammalian cell culture and phospho-Akt Western blotting

3T3-L1 adipocytes were cultured and differentiated as previously described [84]. For siRNA transfections, cells from 4 days post-induction of adipocyte differentiation were used as previously described [85]. Cells were stimulated with increasing concentrations of insulin and isolated proteins were analyzed by Western blotting.

Cell culture and siRNA transfection:

3T3-L1 adipocytes were cultured and differentiated in complete Dulbecco's modified Eagle's medium (10% fetal bovine serum, 50 units/ml penicillin, and 50 g/ml streptomycin) as previously described [84]. For siRNA transfections, cells from 4 days postinduction of adipocyte differentiation were used as previously described (Tang et al., 2006). Briefly, $1.125 \times 10_6$ cells were electroporated using 6 nmol of siRNA and then plated in 5 wells of a 12-well plate. Cells were recovered in complete DMEM and cultured for 48 h after the transfection prior to the experiments.

Insulin stimulation and phospho-Akt western blotting

3T3-L1 adipocytes transfected with siRNA were serum-starved for 18 hours. Cells were stimulated with increasing concentrations of insulin for a period of 30 minutes. Following insulin stimulation, the cells were washed with ice-cold PBS and harvested on ice as described previously [84]. Protein samples were resolved on 8% SDS-PAGE and transferred to a nitrocellulose (NC) membrane. Antibodies used were Phospho-Akt Ser 473 (Cell Signaling, USA; Catalog no. 9271), Phospho-Akt Thr 308 (Cell Signaling, USA; Catalog no. 9275), total Akt antibody (Cell Signaling, USA; Catalog no. 9272). Secondary antibody incubation was performed as above in 1% BSA. Changes in the phosphorylation of Akt pSer 473 and pThr 308 were quantified through densitometry using NIH ImageJ and normalized for loading against the non-phosphorylated total Akt levels.

RNA isolation and real-time PCR

RNA was isolated using Trizol (Invitrogen, USA). Briefly, worms grown on vector, *daf-16*, *daf-18* or *pptr-1* RNAi were washed off the plates with M9 buffer. Next, 0.3 ml of Trizol reagent was added and the worm mixture was vortexed vigorously. The RNA was then purified by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA, the quality of the ribosomal 28 S and 18 S was visually inspected on an agarose gel. Next, cDNA was synthesized using 2 µg of

RNA and the SuperScript cDNA synthesis kit (Invitrogen, USA). Gene expression levels were determined by real time PCR using the SYBR® Green PCR Master Mix and 7000 Real-Time PCR System (Applied Biosystems, USA). Relative gene expression was compared to actin as an internal loading control.

DAPI Staining

pptr-1::mC-flag worms were grown on a plate with OP50 as the food source. Worms were washed off the plate with PBS and rinsed an additional three times with PBS collecting the worms each time by briefly spinning the worms at 3000 rpm for 1 minute. After the final spin, the supernatant was removed and 500µl of 3% Formaldehyde (diluted with potassium phosphate buffer, KH2PO4) was added to the worm pellet. The samples were fixed for 15-20 minutes with gentle shaking, followed by addition of 500µl of PBS-Tween (0.1%) and another gentle mix. The samples were then spun at 3000rpm for 1 minute and washed twice with PBS-Tween and the supernatant was removed. 2µl of DAPI (1mg/mL, Sigma D9542) added to 500µl of PBS and the samples were incubated in this solution for 15-20 minutes before mounting on slides and visualized using the Zeiss Axioscope 2+ microscope.

Table 2.2: List of Strains used in this study

Strains used in this study: N2 (wild type), P*daf-16a::daf-16a::gfp,* CB1370 [*daf-2(e1370)*], DR1572 [*daf-2(e1368)*], RB759 [*akt-1(ok525)*], JT9609 [*pdk-1(sa680)*], VC204 [*akt-2(ok393)*], VC345 [*sgk-1(ok538)*] and PD4251. Strains generated for this study are listed below.

Strain	Strain #	Comment
daf-2(e1370);akt-1(ok525)	HT1547	
daf-2(e1370);akt-2(ok393)	HT1548	
daf-2(e1370);sgk-1(ok538)	HT1648	
daf-2(e1370);daf-3(mgDf90)	HT1607	
akt-1::gfp	HT1632	Integrated *SP209 Pakt-1::akt-1::gfp*, *rol-6* from Ruvkun Lab (Paradis and Ruvkun, 1998)
akt-2::gfp; unc-119(+); unc-119 (ed3)	HT1604	Integrated Line
sgk-1::gfp	HT1634	Integrated BR2777 *Ex[sgk-1::gfp]* from Baumeister Lab (Hertweck et al., 2004)
daf-2(e1370);daf-16::gfp	HT1531	Crossed P*daf-16a::daf-16::gfp* from Ruvkun Lab to *daf-2(e1370)* (Lee et al., 2001)
pptr-1::mC-flag	HT1630	Integrated Line, 4x backcrossed to N2, contains *unc-119(+)* marker
akt-1::gfp; pptr-1::mc-flag	HT1644	
akt-2::gfp; pptr-1::mc-flag	HT1645	
sgk-1::gfp; pptr-1::mc-flag	HT1635	
daf-16::gfp; pptr-1::mc-flag	HT1646	Crossed P*daf-16a::daf-16a::gfp* to *pptr-1::mC-flag*
myo-3::gfp; pptr-1::mc-flag	HT1647	Crossed PD4251 to *pptr-1 ::mc-flag* (Fire et al., 1998)
daf-2(e1370); pptr-1::mc-flag	HT1641	
daf-2(e1370) ; Psod-3 ::gfp	HT1643	Crossed *muIs84[pAD76(sod-3::GFP)]* to *daf-2(e1370)* (Libina et al., 2003)
unc-119(+) ; unc-119(ed3)	HT1638	Extrachromosomal Array Line
daf-2(e1370) ; unc-119(+) ; unc-119(ed3)	HT1642	Extrachromosomal Array Line

Acknowledgements

Preface

This section provides a further perspective on our findings from Chapter 2. We discuss additional aspects of PPTR-1 function and regulation in the context of insulin/IGF-1 signaling.

The following section has been published as:

InAKTivation of Insulin/IGF-1 Signaling by

Dephosphorylation:

A Perspective

Summary

Signal transduction pathways are tightly regulated by phosphorylation-dephosphorylation cycles and yet the mammalian genome contains far more genes that encode protein kinases than protein phosphatases. Therefore, to target specific substrates, many phosphatases associate with distinct regulatory subunits and thereby modulate multiple cellular processes. One such example is the *C. elegans* PP2A regulatory subunit PPTR-1 that negatively regulates the insulin/insulin-like growth factor signaling pathway to modulate longevity, dauer diapause, fat metabolism and stress resistance. PPTR-1, as well as its mammalian homolog B56β, specifically target the PP2A enzyme to AKT and mediate the dephosphorylation of this important kinase at a conserved threonine residue. In *C. elegans*, the major consequence of this modulation is activation of the FOXO transcription factor homolog DAF-16, which in turn regulates transcription of its many target genes involved in longevity and stress resistance. Understanding the function of B56 subunits may have important consequences in diseases such as Type 2 diabetes and cancer where the balance of Akt phosphorylation is deregulated.

Introduction

Recent studies have identified B56 regulatory subunit of PP2A as a critical modulator of insulin/IGF-1 signaling in the nematode *Caenorhabditis elegans* (*C. elegans*) and mammalian cells as well as in the fruitfly *Drosophila melanogaster* [86,87]. Here we discuss how this regulatory subunit directs the otherwise broadly expressed PP2A to Akt and regulates its dephosphorylation at its conserved Threonine residue [86]. In *C. elegans*, this results in changes in longevity, fat metabolism, dauer diapause and stress resistance [86]. The insulin/IGF-1 signaling pathway is structurally as well as functionally conserved between nematodes, flies and higher organisms such as rodents and humans (Figure 2.6)[4]. In *C. elegans*, the insulin/IGF-1 signaling pathway regulates longevity, dauer diapause, fat metabolism and stress resistance [1,2,3]. This pathway is typically termed insulin/IGF-1 signaling as the receptor in the pathway, encoded by the gene *daf-2*, is equally related to both, the mammalian insulin receptor and insulin-like growth factor receptor [5]. Further, the ligand for this receptor has not been verified biochemically.

Downstream of the *daf-2* receptor is a phosphatidylinositol (PI) 3-kinase signaling pathway that ultimately regulates the major target of this cascade, the Forkhead transcription factor box O (FOXO) homolog *daf-16* [11]. In mammals and worms, PI 3-kinase activation results in the conversion of membrane phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2) to phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3) [88]. In mammals and presumably in worms,

PI(3,4,5)P3 phosphoinositides recruit phosphoinositide-dependent kinases such as phosphoinositol-dependent kinase 1 (PDK-1) and Akt to the plasma membrane through their pleckstrin homology (PH) domains, subsequently leading to their activation [89].

C. elegans has two different AKT proteins, AKT-1 and AKT-2 [9]. In addition, the serum- and glucocorticoid-inducible kinase 1 (SGK1), which is closely related to Akt, has been shown to act at the same level as Akt in the pathway [90]. Similar to mammals, in C. elegans activated AKT-1/2 and SGK-1 can phosphorylate DAF-16/FOXO on distinct serine/threonine residues, and this leads to its inactivation as well as cytoplasmic sequestration by the 14-3-3 proteins (Figure 2.6) [11]. Under low insulin-signaling conditions, DAF-16/FOXO is able to translocate into the nucleus and transactivate or repress its many target genes. Several genome-.wide studies have revealed antioxidant genes, molecular chaperones, detoxification genes, antimicrobial genes and metabolic genes as direct or potential targets of DAF-16 [58]. These target genes may regulate longevity, stress-resistance, dauer diapause and metabolism in a combined manner.

Given that insulin/IGF-1 signaling regulates multiple cellular processes, tight regulation of kinase activity at each step is important to maintain the threshold of signals to elicit appropriate cellular responses. While many of the kinases in insulin/IGF-1 signaling have been well characterized, little is known about the phosphatases that downregulate signals through the pathway. The most well

studied negative regulator of insulin/IGF-1 signaling is the lipid phosphatase and tensin homolog PTEN [91]. In *C. elegans*, the PTEN homolog DAF-18 negatively regulates PI3-kinase signaling to ultimately promote DAF-16 nuclear localization and function. Loss-of-function mutations or knockdown by RNA interference of *daf-18* results in stress resistance, dauer suppression and reduced fat storage [86].

PPTR-1/B56 regulates Insulin/IGF-1 Signaling

We reasoned that there would be additional phosphatases in the pathway in addition to DAF-18/PTEN to counterbalance the effects of the kinases. We performed a directed RNAi screen in *C. elegans* that assessed the role of 60 putative serine/threonine phosphatases in modulating the insulin/IGF-1 signaling pathway by using dauer diapause as a readout [86]. We also included six regulatory subunit genes of the PP2A holoenzyme (explained further below). In the screen, we assayed for the contribution of the different serine/threonine phosphatases on *daf-2* dauer formation. Our positive control was *daf-18* RNAi, as we were primarily interested in genes that functioned as negative regulators of the pathway. We did, however, identify several genes that when knocked down by RNAi, resulted in enhanced dauer formation. These may represent phosphatases that act as activators of the pathway or even positive regulators of kinase activity. Among the negative regulators identified in the screen, our top candidate was the gene *protein phosphatase two A (2A) regulatory subunit-1*
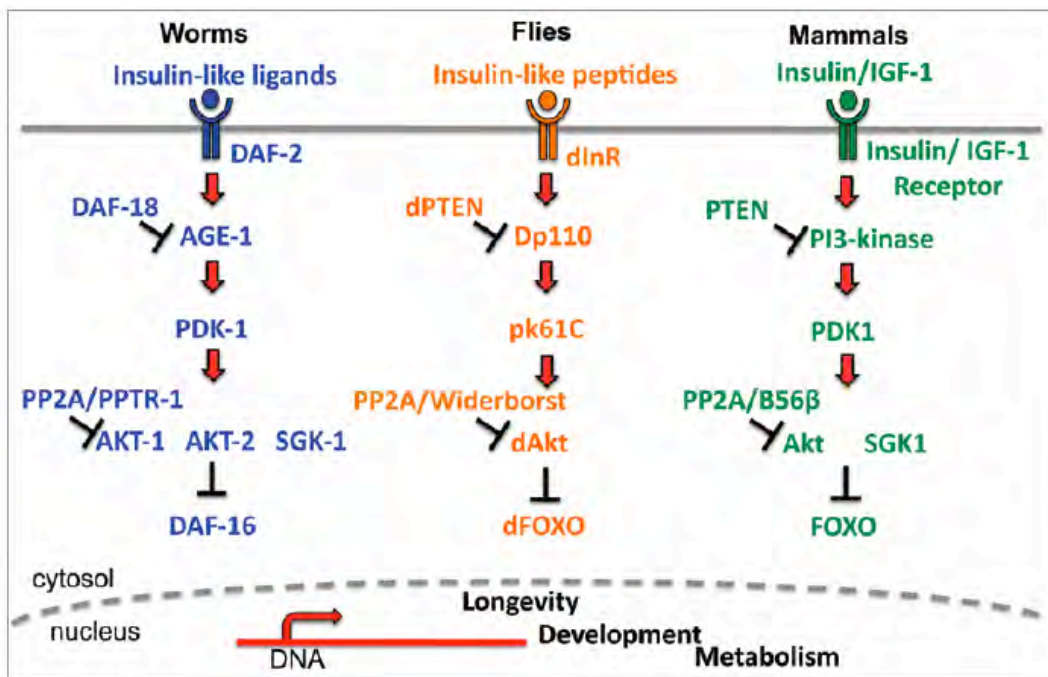
Figure 2.6: PPTR-1/B56ß is a conserved regulator of insulin/IGF-1 signaling

(pptr-1) [86]. Knockdown of *pptr-1* by RNAi resulted in robust suppression of dauer formation similar to *daf-18* RNAi.

In addition to dauer diapause, the insulin/IGF-1 pathway also regulates longevity, stress resistance and fat metabolism [2]. Phenotypic analyses revealed that *pptr*-1 was a robust modulator of these additional outputs. Indeed, while *pptr-1* RNAi resulted in a reduction in the lifespan of long-lived insulin/IGF-1 receptor (*daf-2)* mutants, overexpression of *pptr-1* conferred almost 30% extension in lifespan [86]. Further, *pptr-1* RNAi resulted in decreased thermotolerance as well as decreased fat storage in *daf-2* mutants, but not in wild type animals. Similarly, a recent screen performed in the fruitfly *Drosophila melanogaster* to identify phosphatases that function in the insulin/IGF-1 signaling pathway identified the *pptr-1* homolog *widerborst* as negative regulator of the pathway and consistent with our findings, *widerborst* was found to be an important regulator of fat metabolism [87].

As mentioned earlier, *pptr-1* encodes a regulatory subunit of the PP2A holoenzyme. PP2A is an abundant serine/threonine protein phosphatase that functions as a holoenzyme consisting of a 36 kDa invariant catalytic core, a 65kDa structural scaffold and a variable regulatory subunit [92]. PP2A has been implicated in several cellular processes such as insulin signaling, cell cycle progression and translation [93]. In our screen, we found that RNAi knockdown of the PP2A catalytic and structural subunits in *C. elegans* resulted in lethality, substantiating the fact that knockdown of such a broadly required phosphatase

produces detrimental effects on multiple cellular functions. Importantly, it is the association of the catalytic and structural core with distinct regulatory subunits that enables the PP2A holoenzyme to modulate multiple processes and yet retain substrate specificity [92,94,95].

There are three predominant families of mammalian PP2A regulatory subunits: the B/B55/PR55, B'/B56/PR61 and B''/PR72 families [92] and currently at least 15 human genes have been identified that encode distinct regulatory subunits [96]. Further, these genes may have additional splice forms that vastly increase the total number of regulatory subunits that associate with the catalytic and structural subunits, ultimately providing substrate specificity as well as distinct spatio-temporal localization within the cell. Post-translational modifications such as methylation and phosphorylation of the C-terminal tail of the PP2A catalytic subunit can affect the binding of members of the B subunit to the holoenzyme [64]. This may constitute additional level of regulation to achieve substrate specificity.

C. elegans has at least seven genes that encode for regulatory subunits and they fall into each of the three PP2A regulatory subunit families (www.wormbase.org) [86]. *pptr-1* belongs to the B56 family of PP2A regulatory subunits. In our studies, we examined six out of the seven regulatory subunits and found that only *pptr-1*, and none of the other five genes, showed significant effects on dauer formation, thus highlighting a specific role for this regulatory subunit [86]. Tissue-expression patterns of these regulatory subunits indicate that

they are not as broadly expressed, spatially or temporally, as the catalytic subunit of PP2A. Indeed, we found that PPTR-1/B56β was only expressed in discrete sets of tissues, including the spermatheca, vulva and several neurons in the worm (Figure 2.7)[86]. In *Drosophila* as well as mammals, members of the B56 family show distinct sub-cellular localization patterns as well, with B56α, B56β and B56ϵ showing cytosolic expression while B56γ shows both, nuclear as well as cytosolic expression [63,87]. In agreement with this finding, DAPI staining and confocal microscopy showed that *C. elegans* PPTR-1/B56β was predominantly cytosolic [86].

PPTR-1/PP2A and Akt

To investigate how a single regulatory subunit, PPTR-1, was able to modulate multiple outputs of insulin/IGF-1 signaling, we performed genetic epistasis analyses with mutants in the insulin/IGF-1 signaling pathway. Epistasis analyses using dauer formation as a readout of the insulin/IGF-1 signaling pathway revealed that *pptr-1* acts downstream of *pdk-1*, at the level *akt-1* [86]. Knockdown of *pptr-1* by RNAi could strongly suppress dauer formation of *daf-2*, and *pdk-1* single mutants as well as *daf-2; akt-2* and *daf-2; sgk-1* double mutants. However, *pptr-1* RNAi had no effect on the dauer formation of *daf-2; akt-1* double mutants, and therefore, we concluded that there was a genetic interaction between *pptr-1* and *akt-1*.

Akt belongs to the AGC family of protein kinases that also include Protein Kinase A, C, ribosomal S6 kinase (S6k) and SGK1 [74]. Akt has been shown to be at the crossroads of several signaling cascades such that active Akt is a regulator of cell cycle progression, cell survival, glucose metabolism as well as protein synthesis [67,97]. Mammalian studies have shown that activation of Akt is achieved through the phosphorylation of two main residues, Threonine 308 and Serine 473 [53,67,97]. While the Thr308 residue is phosphorylated by the PDK-1 kinase, the mammalian target of rapamycin (mTOR) complex 2 (TORC2) phosphorylates Serine 473 [53]. At the protein level, *C. elegans* AKT-1 and AKT-2 share nearly 60% sequence homology [9]. Interestingly, AKT-1 contains both the Thr 350 (mammalian 308) as well as the Serine 517 (mammalian 473) residues, whereas AKT-2 lacks the C-terminal serine residue [9,86]. Tissue expression analyses show that AKT-1 and AKT-2 share overlapping expression in multiple tissues [9,86].

In *C. elegans*, AKT-1, AKT-2 and SGK-1 can form a complex to negatively regulate DAF-16 by direct phosphorylation [10]. However these kinases show phenotypic differences as well. Reduction of function mutations or RNAi of *akt-1* and/or *akt-2* results in enhanced dauer formation as well as lifespan extension [9]. Mutation or RNAi of *sgk-1* has been shown to either increase or decrease lifespan and have a minor effect on dauer formation [10]. As shown in Figure 2.7, there are also differences in the expression patterns in *C. elegans* for AKT-1, AKT-2 and SGK-1.

Humans have three Akt proteins, Akt1, Akt2 and Akt3 that are encoded by distinct genes and share nearly 80% sequence homology [98]. Based on homology, *C. elegans* AKT-1 is more related to mammalian Akt2 while *C. elegans* AKT-2 shows homology to mammalian Akt3. Studies using gene knockouts in mice have revealed more specific roles for each Akt isoform: *Akt1* null mice are small and show defects in placental development. In contrast, *Akt2* null mice show severe defects in glucose metabolism including insulin resistance and age-dependent loss of adipose tissue while *Akt3* null mice show a reduced brain size [98]. Consistent with the role of Akt in growth and cell survival, all three Akt mutants show greatly reduced cell size as well as mass [98,99].

Our genetic epistasis studies showed that *pptr-1* acted at the level of *akt-1* but not on the closely related *akt-2* or *sgk-1*. Tissue expression analyses revealed a remarkable overlap in the expression of PPTR-1 with AKT-1, partial overlap with AKT-2 and little or no overlap with SGK-1, pointing at the specificity of the *pptr-1/akt-1* interaction (Figure 2.7) [86]. Consistent with this, in *Drosophila*, genetic epistasis analysis placed *widerborst* within the PI 3-kinase pathway at the level of *Akt1* [87]. From these results, we hypothesized that PPTR-1 was modulating insulin/IGF-1 signaling and DAF-16 activity by regulating AKT-1 phosphorylation. Using affinity-purified phospho-specific antibodies raised against each of the two AKT phosphorylation sites in *C. elegans*, we showed that PPTR-1 modulated Thr 350 dephosphorylation, and to a lesser extent, Ser 517. This interaction was then verified in mammalian 3T3-L1 adipocytes, where mammalian B56β but not other

B56 regulatory subunits robustly regulated Akt dephosphorylation at Thr308 [86] and the Ser 473 site was unaffected. In *Drosophila*, Widerborst interacts with Akt1 and regulates its dephosphorylation in a PP2A-dependent manner [87]. These phosphorylation experiments highlight the remarkable conservation of the insulin/IGF-1 signaling pathway in terms of regulation and functionality between worms, flies and mammals. Importantly, regulation of AKT phosphorylation is critical in humans as well. Reduced AKT phosphorylation has been associated with insulin resistance in patients with type 2 diabetes and hyperphosphorylated AKT is common in cancers where PTEN is mutated [74].

Recent mammalian studies identified the PH domain leucine rich repeat protein phosphatases (PHLPP), members of the PP2C family as important regulators of Ser 473, but not Thr 308 of Akt [71,100]. Specifically, PHLPP1 can dephosphorylate Akt1 and Akt3 at Ser 473, while PHLPP2 can dephosphorylate Akt2 and Akt3 at Ser 473 [100]. As a consequence, these two phosphatases elicit different outputs of Akt signaling such as cell cycle control and glycogen metabolism respectively. The PHLPP homolog in *C. elegans* did not affect dauer formation in our screen (Padmanabhan and Tissenbaum, unpublished data)**.** Consistent with this, our mammalian data showed that siRNA of B56ß or PP2A in insulin-stimulated 3T3-L1 adipocytes resulted in enhanced Akt Thr308 phosphorylation but had no effect on Ser473 phosphorylation [86]. Together, these studies reveal how distinct phosphatases can dephosphorylate two distinct

residues within a single protein, thereby achieving a remarkable level of complexity in the modulation of signal transduction pathways.

PPTR-1/B56 modulates DAF-16/FOXO activity

What are the functional consequences of PPTR-1-dependent modulation of AKT-1 in *C. elegans*? The major output of *C. elegans* insulin/IGF-1 signaling is the negative regulation of DAF-16/FOXO [2]. Dosage modulation of PPTR-1 had opposite effects on DAF-16 nuclear localization: while *pptr-1* RNAi resulted in more cytosolic and inactive DAF-16, *pptr-1* overexpression enhanced DAF-16 nuclear localization as well as the lifespan of the worms [86]. Similarly, overexpression of *widerborst* in flies results in a reduction in the adult eye, a phenotype similar to *dFoxo* overexpression and co-overexpression of both genes results in the enhancement of the *dFoxo* overexpression phenotype [87]. Therefore, both of these studies show that modulation of the PPTR-1/B56β dosage can affect FOXO-dependent phenotypes.

Phosphorylation of FOXO by Akt at three serine/threonine residues is an important determinant of its sub-cellular localization in mammals [101]. In addition, DAF-16/FOXO is also positively regulated by JNK, MST-1 and AMP-dependent kinase (AMPK) through phosphorylations at separate residues [3,13]. Therefore, there may be phosphatases that directly dephosphorylate and activate/inhibit DAF-16/FOXO itself. In a genome-wide screen for kinases and phosphatases that modulated dFOXO subcellular localization, activity and protein

stability in *Drosophila* S2 cells, several kinases were identified, including many well-known regulators such as JNK and AKT1, but few phosphatases were identified [102]. Since kinases can have both stimulatory and inhibitory functions and multiple phosphorylation sites exist, the identification of a DAF-16/FOXO phosphatase(s) may require a more detailed approach. For example, the sensitization of the insulin/IGF-1 pathway, the type of stress or metabolic state (fed versus starved) may result the association of DAF-16/FOXO proteins with distinct phosphatases. Given that DAF-16/FOXO nuclear-cytosolic localization is so dynamic, the interaction with the phosphatase(s) may be transient and difficult to capture. Identification of phosphatases that directly modulate DAF-16/FOXO function will not only provide a better perspective on the hundreds of genes that FOXO proteins transcriptionally activate/repress, but also have implications in our understanding of FOXOs in disease [103] [104].

Future directions

Several questions regarding PPTR-1/B56 and its regulation of the insulin/IGF-1 pathway via AKT dephosphorylation stem from these findings.

1) Regulation of PPTR-1

We found that PPTR-1 function was more important under low signaling conditions. This was evident by the fact that *pptr-1* RNAi reduced the lifespan, fat storage and thermotolerance of *daf-2* mutant worms but did not affect wild type

worms. It is possible that under low insulin/IGF-1 signaling, PPTR-1 functions to sensitize the pathway even further to ultimately promote survival. In the context of mammals, it will be intriguing to further study the role of PPTR-1/B56, and determine if levels of blood glucose (or more broadly, nutritional status) modulate the activity of this protein. Studies have shown that PP2A itself is downregulated under normal insulin signaling conditions [66].

Similarly, PPTR-1 itself, may also be regulated either at the transcriptional level or posttranscriptionally. Indeed in cardiomyocytes, an increase in wild type or constitutively active FOXO resulted in a corresponding decrease in PP2A activity and subsequent activation of Akt, thereby indicating a feedback loop [105]. Therefore, further studies will help to identify the upstream cues that activate or repress PPTR-1/B56 activity.

2) The role of other subunits

Our studies in *C. elegans* show that PPTR-1 modulates multiple processes associated with insulin/IGF-1 signaling. Importantly, given that AKT is at the focal point of several signaling pathways, the question that arises is whether PPTR-1 is a broad regulator of AKT activity, or does it specifically play a role in insulin-dependent activation of AKT? The other regulatory subunits did not significantly affect dauer diapause, but are likely to modulate other PP2A-dependent processes, even possibly other outputs of the pathway such as lifespan, stress resistance and fat metabolism. Biochemical approaches such as

immunoprecipitation followed by mass spectrometry may help to identify the *C. elegans* substrates for these other regulatory subunits and PP2A.

3) <u>Tissue-specificity</u>

A third aspect that would be interesting to investigate further is the tissue-specific regulation of PPTR-1/B56. Although PPTR-1 is expressed in a subset of tissues, modulation of PPTR-1 dosage resulted in changes in organismal longevity suggesting that cell non-autonomous regulation and neuroendocrine signaling is important for this function (Figure 2.7). In addition to the head neurons, PPTR-1 shares remarkable overlap with AKT-1 in the spermatheca and vulva. Interestingly, AKT-1::GFP[9,86] and PPTR-1::GFP [86] did not show any expression in the intestine, the major tissue for fat storage in the worm. Moreover, tissue-specific studies have shown that the intestine is the most important tissue for DAF-16-dependent regulation of lifespan [62]. Akt-dependent phosphorylation is the major mechanism by which the activity of DAF-16/FOXO is regulated. Therefore, how and when does the direct regulation of AKT and DAF-16 occur? Further studies are necessary in worms as well as mammals to determine how PPTR-1/B56 regulates insulin/IGF-1 signaling in the context of the whole organism.

All of our expression studies and biochemistry experiments in *C. elegans* used overexpression strains, as we were unable to pull-down endogenous AKT-1. It is possible that there are low levels of expression that we could not detect in

the intestine or that the AKT-1::GFP strain for some reason was not expressed in the intestine. We found that SGK-1::GFP and PPTR-1::mCherry-FLAG showed no overlap in their expression, and yet AKT-1, AKT-2 and SGK-1 have been shown biochemically to form a complex to regulate DAF-16 activity [10]. It is unclear if the tagged versions of these proteins entirely phenocopy the roles of native proteins and further experiments such as immunostaining with antibodies to target endogenous AKT, SGK-1 and PPTR-1 may provide a better understanding of their expression patterns, interaction and regulation. Finally, we identified a number of additional candidates in our RNAi screen that could potentially be important regulators of insulin/IGF-1 signaling. The phosphatases that negatively regulate PDK-1, AKT-2, SGK-1 and DAF-16 itself are currently unknown and identification of these would provide us with a much better understanding of the regulation of this important pathway.

Taken together, the PPTR-1/B56 regulatory subunit of PP2A is a novel and robust modulator of the insulin/IGF-1 signaling pathway. By regulating the dephosphorylation of a conserved threonine residue on Akt, PPTR-1/B56 can activate DAF-16/FOXO and positively regulate its transcriptional activity. The genes that are up/downregulated as a consequence are likely to play a combinatorial role in regulating longevity, stress resistance, dauer diapause and fat metabolism. Given its extensive conservation and the key role AKT-1 plays in mammals, further studies on PPTR-1/B56 could be of critical importance for diseases, such as cancer and diabetes.

Tissue	Insulin/IGF-1 signaling component
Head neurons	AKT-1, PPTR-1, SGK-1[a]
Pharynx	AKT-1, AKT-2, PPTR-1
Intestine	DAF-16[b], SGK-1
Vulva	AKT-1, AKT-2, PPTR-1
Spermathecae	AKT-1, AKT-2, PPTR-1
Tail Neurons	AKT-1, PPTR-1, SGK-1[a]

[a] SGK-1 is expressed in neurons distinct from AKT-1 and PPTR-1
[b] DAF-16 is expressed in multiple tissues but the intestine is the most important tissue for longevity
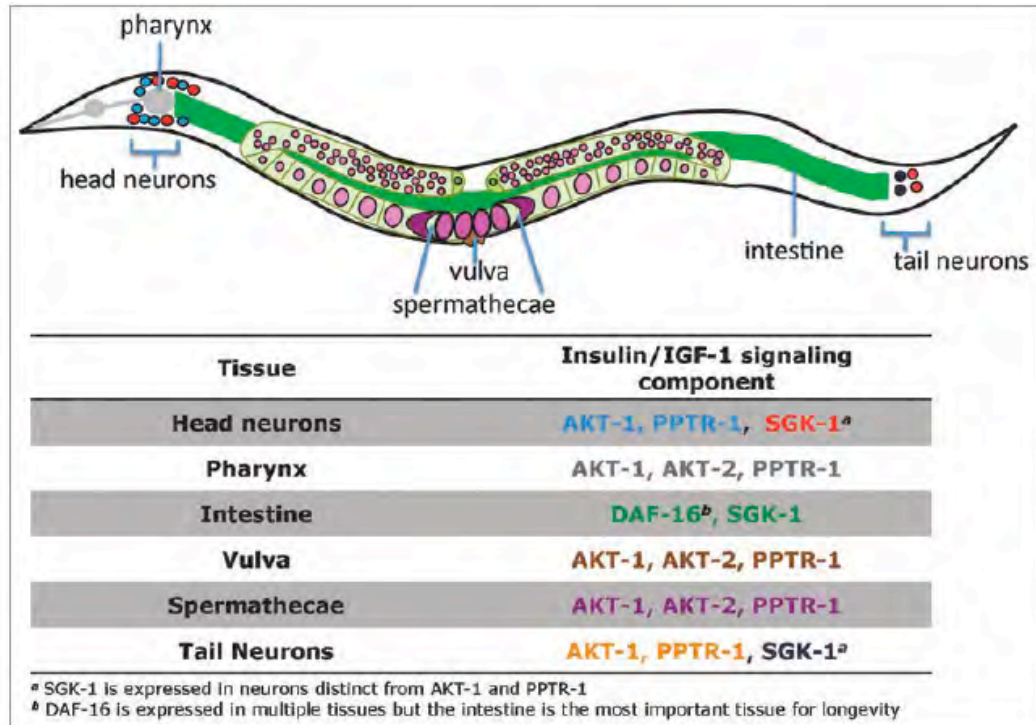
Figure 2.7: Overlapping and distinct expression patterns of insulin/IGF-1 signaling components suggest a possible neuroendocrine regulation of the pathway.

Acknowledgements

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Preface to Chapter 3

This chapter describes the characterization of the phosphatase PDP-1 and its role in modulating the insulin/IGF-1 signaling pathway through negative regulation of TGF-β signaling. The work in the following chapter was a collaborative effort. Ankita Bansal, a graduate student in the lab, did the fat staining assays in Figure 3.2 and dauer assays in Figure 3.3. I performed all the other experiments in this chapter. This chapter is a part of the following manuscript that Dr. Heidi Tissenbaum and I wrote that has been submitted for publication:

Narasimhan SD, Yen K, Bansal A, Padmanabhan S and Tissenbaum HA (2010). PDP-1 Regulates Lifespan, Fat and Development through the Insulin/IGF-1 and TGF-β signaling pathway (Submitted).

Chapter 3:

PDP-1 Regulates Lifespan, Fat and Development

through the Insulin/IGF-1 and TGF-β Signaling Pathways

Summary

The insulin/IGF-1 signaling (IIS) pathway is a conserved regulator of longevity, development and metabolism. In *C.elegans,* IIS involves activation of DAF-2 (insulin/IGF-1 receptor tyrosine kinase), AGE-1 (PI3-kinase) and additional downstream serine/threonine kinases that ultimately phosphorylate and negatively regulate the single FOXO transcription factor homolog DAF-16. Phosphatases help to maintain cellular signaling homeostasis by counterbalancing kinase activity. However, few phosphatases have been identified that negatively regulate IIS pathway.

Here we identify and characterize PDP-1 as a novel negative modulator of the IIS pathway. We show that PDP-1 regulates multiple outputs of IIS such as longevity, fat storage and a developmental stage known as dauer. In addition, PDP-1 promotes DAF-16 nuclear localization as well as transcriptional activity. Interestingly, genetic epistasis analyses place PDP-1 in the DAF-7/TGF-β signaling pathway, at the level of the R-SMAD proteins DAF-14 and DAF-8. The DAF-7/TGF-β signaling pathway regulates insulin gene expression in worms and consistent with this, we find that PDP-1 modulates the expression of several insulin genes that are likely to feed into the IIS pathway to regulate DAF-16 activity.

Dysregulation of IIS and TGF-signaling has been implicated in diseases such as Type 2 diabetes, obesity and cancer. Our results may provide a new

perspective in our understanding of the regulation of these pathways under normal conditions and in the context of disease.

Introduction

Insulin/IGF-1 signaling (IIS) is a conserved neuroendocrine pathway that regulates longevity, development and energy metabolism across phylogeny [1,2]. In the roundworm *Caenorhabditis elegans*, activation of the DAF-2 insulin/IGF-1 receptor tyrosine kinase intiates an AAP-1/AGE-1 PI 3-kinase signaling cascade involving the downstream serine/threonine kinases PDK-1, AKT-1, and AKT-2 [3,4,5,6,7]. Activated AKT-1 and AKT-2 phosphorylate DAF-16, the single Forkhead O family transcription factor homolog in *C.elegans* [8]. Phosphorylation of DAF-16 results in its inactivation and sequestration in the cytosol [9,10]. Under low signaling conditions, DAF-16 translocates into the nucleus, where it can transactivate/repress hundreds of target genes [9,10,11,12,13].

The dauer is an alternative survival stage that worms can enter upon poor environmental conditions and crowding [14]. Mutations in the kinases upstream of DAF-16 result in an increase in lifespan, dauer formation, fat storage and/or stress resistance, and loss-of-function mutations in *daf-16* completely suppress these phenotypes [15,16,17,18]. Besides the IIS pathway, dauer formation in *C.elegans* is also regulated by a TGF-β like pathway [19,20,21]. Activation of TGF-β signaling is achieved through binding of the DAF-7 BMP-like ligand to the DAF-1/DAF-4, the Type I/II receptors, which phosphorylate and activate the downstream receptor-associated SMAD (R-SMAD) proteins DAF-8 and DAF-14. Under normal signaling conditions, these SMADs act to antagonize the transcriptional activity of the DAF-3 Co-SMAD and the DAF-5 SNO-SKI repressor

[22,23,24,25,26,27]. Reduction of function mutations in *daf-7, daf-1, daf-4, daf-8* and *daf-14* show temperature-sensitive constitutive dauer-formation and mutations in *daf-3* and/or *daf-5* completely suppress this phenotype [21,28]. The DAF-7/TGF-β is thought to act in a parallel manner with IIS to modulate dauer formation [29,30,31].

   The PTEN lipid phosphatase homolog DAF-18, which antagonizes signaling at the level of AGE-1/PI3-kinase, is a major negative regulator of IIS. In contrast to the kinases, loss-of-function mutations in *daf-18* result in a reduction in lifespan, fat storage, dauer formation and stress resistance [30,32,33,34,35,36]. We recently performed a directed RNA interference (RNAi) screen for serine/threonine phosphatases that regulate *C.elegans* IIS using dauer formation as an output [37]. We identified the PP2A regulatory subunit PPTR-1 as an important regulator of AKT dephosphorylation as well as of DAF-16-dependent phenotypes [37]. Here we characterize PDP-1, another candidate from this screen. Dosage modulation of PDP-1 regulates multiple outputs of the IIS pathway. PDP-1 shares significant homology with mamalian pyruvate dehydrogenase phosphatase (PDP). PDP-1 is an important metabolic enzyme that positively regulates the pyruvate dehydrogenase enzyme complex (PDHc). To our surprise, RNAi of components of the PDHc in worms have little effect on dauer formation and other outputs of the pathway. Interestingly, we report that PDP-1 acts in the DAF-7/TGF-β pathway but is a robust modulator of IIS as well as DAF-16 activity. Through these studies, we suggest that IIS and TGF-β

signaling are more tightly connected than previously appreciated and we point to

the insulins as a potential mediator of the crosstalk between these two pathways.

Results

1. *C.elegans* PDP-1 regulates *daf-2* dauer formation independent of PDH

Our RNAi screen was designed to identify serine/threonine phosphatases that modulated dauer formation of *daf-2(e1370)*, a temperature-sensitive mutant of the insulin/IGF-1 receptor *daf-2* [38]. We were particularly interested in phosphatases that would negatively regulate IIS similar to DAF-18/PTEN, and for all RNAi based assays described below, *daf-18* RNAi was used as a positive control [37]. From this screen, we identified *pdp-1* as a modulator of *daf-2(e1370)* dauer formation (Fig 3.1A and Fig 3.3). We find that *pdp-1* RNAi significantly reduces dauer formation of *daf-2(e1370)* worms, similar to *daf-18* RNAi (Figure1). This phenotype is not allele-specific, as *pdp-1* RNAi results in suppression of dauer formation in a different allele of *daf-2, daf-2(e1368)* (Figure 3.1B and Fig 3.3). Similar to the results with the RNAi, a mutation in *pdp-1* also affects dauer formation - *pdp-1(tm3734); daf-2(e1370)* double mutants form significantly fewer dauers when compared with the *daf-2(e1370)* parental strain (Figure 3.3).

The gene *pdp-1* bears approximately 54% homology with fly and mammalian pyruvate dehydrogenase phosphatase (PDP), based upon BLAST search results. Given its homology to PDP in higher organisms, we wondered whether the effect of *pdp-1* knockdown on *daf-2* dauer formation was a consequence of modulating the activity of the pyruvate dehydrogenase enzyme complex (PDHc). The PDHc is a multi-subunit enzyme complex consisting of three major enzymes:

E1 pyruvate dehydrogenase, E2 dihydrolipoyl acetyltransferase and E3 dihydrolipoyl dehydrogenase that regulate energy metabolism [39]. Active PDHc converts pyruvate to acetyl-coA, which can either enter the TCA cycle or be used for fatty acid synthesis. In mammals, regulation of PDHc activity is primarily achieved through reversible phosphorylation/dephosphorylation of the E1α subunit by pyruvate dehydrogenase kinase (PDHK) and pyruvate dehyrogenase phosphatase (PDP), with phosphorylation inactivating the enzyme complex [39].

All of the components of the PDH complex have conserved *C.elegans* homologs, encoded by the genes T05H10.6 (E1α), C04C3.3 (E1β), F23B12.5 (E2), LLC1.3 (E3), *pdhk-2* (PDHK) and *pdp-1* (PDP). To test whether modulation of PDHc activity affects *daf-2* dauer formation, we grew *daf-2(e1370)* worms on PDHc RNAi. We observed significant reduction in the transcripts of the PDHc components with our RNAi clones (Figure 3.1H). To our surprise, RNAi of the E1α subunit had no effect on *daf-2* dauer formation, while *pdp-1* RNAi resulted in dauer suppression (Figure 3.1C). In addition, RNAi of both the E1 subunits, E1α and E1β, or the E2 subunit did not affect *daf-2* dauer formation (Figure 3.1). Knockdown of the E3 subunit resulted in lethality (data not shown). Interestingly, *pdhk-2* RNAi resulted in slight suppression *daf-2(e1370)* dauer formation (Figure 3.1) but this was observed to be an allele-specific effect as *pdhk-2* RNAi had no effect on dauer formation of *daf-2(e1368)* as well as on *pdk-1(sa680)* mutants (Figure 3.1D & E).

In addition, we did not observe significant overlap between the tissue expression patterns of PDP-1 with those of the E1 and E2 subunits (Figure 3.1). PDP-1 expression was enriched in the head muscle, several neurons in the head and pharynx, intestine and tail neurons. We did not observe any expression in the pharynx. In contrast, E1 and E2 expression was observed throughout the body of the worm but was significantly enriched in the pharynx. Based upon our genetic and expression data, PDP-1 modulates *daf-2* dauer formation and this function is likely to be independent of its role in regulating the PDHc.

G
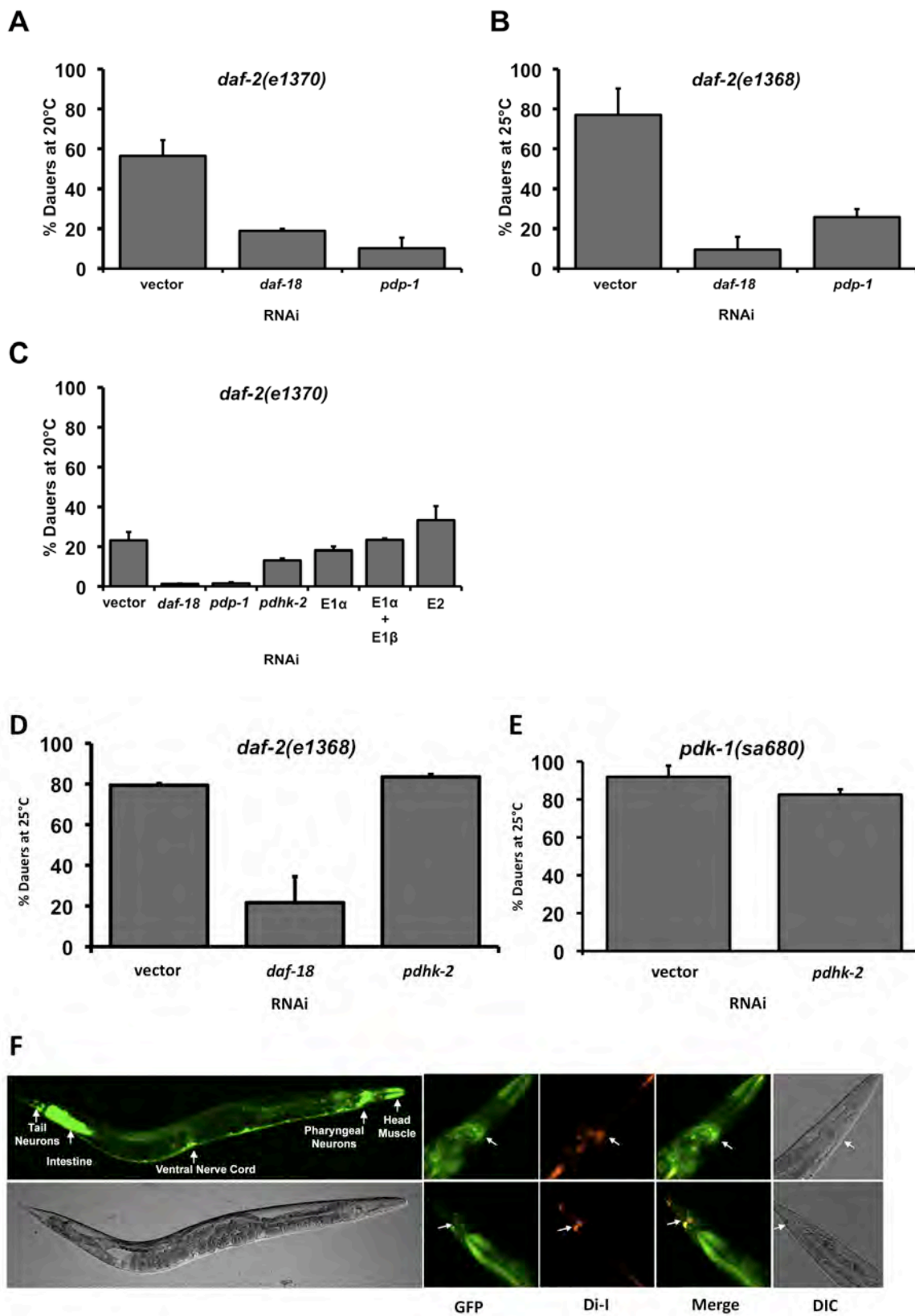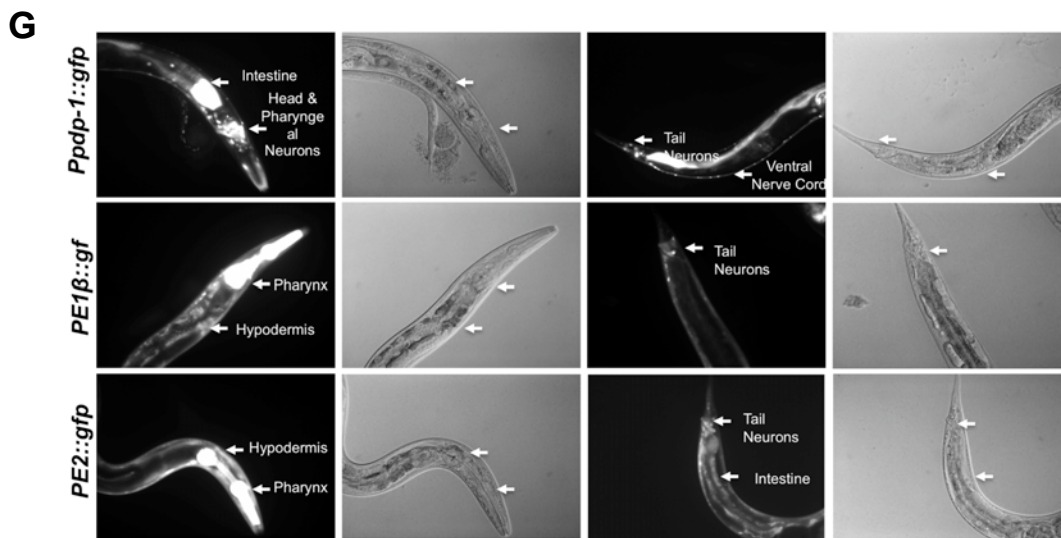
H

Transcripts determined by Q-PCR (% of vector RNAi)		
RNAi	**Set 1**	**Set 2**
T05H10.6 (E1α)	**0.1**	**0.1**
C04C3.3 (E1β)	**0.1**	**0.2**
F23B12.5 (E2)	**0.2**	**0.4**
LLC1.3 (E3)	**0.4**	**0.2**
pdhk-2	**0.4**[a]	**0.1**[a]
pdp-1	**0.3**	**0.2**
daf-18	**0.1**	**0.3**

[a] The experiments for *pdhk-2* RNAi were performed independently

Figure 3.1: PDP-1 regulates *daf-2* dauer formation independent of the PDHc

Error bars indicate the standard deviation among the different RNAi plates within one experiment. Data shown are from one representative experiment in all the assays except for Figure 3.1H

A) *pdp-1* RNAi suppresses *daf-2(e1370)* dauer formation similar to *daf-18* RNAi. Dauer formation of *daf-2(e1370)* was 56.5 ± 8.0 % (n=278) on vector RNAi, 18.9 ± 0.8 % (n=79) on *daf-18* RNAi ($p < 0.05$) and 10.5 ± 5.3 % (n=293) on *pdp-1* RNAi ($p < 0.05$).

B) *pdp-1* RNAi suppresses dauer formation of *daf-2(e1368)* worms similar to *daf-18* RNAi. Dauer formation of *daf-2(e1368)* was 77.1 ± 13.2 % dauers (n=297) on vector RNAi, 9.4 ± 6.4 % (n=258) dauers on *daf-18* RNAi ($p < 0.06$) and 25.9 ± 3.9 % (n=636) dauers on *pdp-1* RNAi ($p < 0.05$).

C) RNAi of other components of the PDHc including the E1α subunit does not affect *daf-2(e1370)* dauer formation. Dauer formation of *daf-2(e1370)* on PDHc RNAi was 23.3 ± 4.1 % (n=282) on vector RNAi, 1.3 ± 0.2 % (n=219) on *daf-18* RNAi ($p < 00.04$), 1.6 ± 0.6 % (n=185) on *pdp-1* RNAi ($p < 0.03$), 13.1 ± 1.0 % (n=233) on *pdhk-2* on RNAi ($p < 0.05$), 18.2 ± 2.0 % (n=193) on E1α RNAi, 23.5 ± 0.5 % (172) on a combination of E1α and E1β RNAi and 33.3 ± 7.1 % (n=25) on E2 RNAi.

D) *pdhk-2* RNAi has no effect on dauer formation of *daf-2(e1368)* mutants. Dauer formation was 79.4 ± 0.9 % (n=504) on vector RNAi, 21.6 ± 12.8 % (n=617) on *daf-18* RNAi and 70.0% ± 9.8 % (n=645) on *pdhk-2* RNAi.

E) *pdhk-2* RNAi has no effect on dauer formation of *pdk-1(sa680)* mutants. Dauer formation was 91.9 ± 5.9 % (n=458) on vector RNAi and 82.5 ± 2.7 %

F) Expression pattern of *pdp-1* as visualized using a *Ppdp-1::gfp* transcriptional fusion strain. Di-I staining shows co-localization in amphid neurons in the head and tail.

G) The *Ppdp-1::gfp* strain does not show complete overlap with the expression patterns of transcriptional fusion strains of the PDHc, *PE1β::gfp* and *PE2::gfp*.

H) Quantitative PCR experiments from two independent repeats showing the knockdown of components of the PDHc by RNAi.

2. PDP-1 regulates multiple outputs of the IIS pathway

In addition to dauer formation, the IIS pathway also regulates longevity, stress resistance and fat storage [17,18]. Mutations in *daf-2* and *age-1* result in a significant extension in lifespan, enhanced resistance to various stresses and increased fat storage [7,33,40,41,42,43]. These phenotypes are suppressed by loss-of-function mutations in *daf-18* and *daf-16* [30,32,33]. We therefore investigated whether dosage modulation of PDP-1 would affect additional outputs of the pathway. We first tested the role of PDP-1 in regulating lifespan (Figure 3.2). The lifespan of wild type worms was not significantly reduced on *pdp-1* RNAi when compared to vector RNAi (Figure 3.2A). However, *pdp-1(tm3734)* mutants show a slightly reduced lifespan relative to wild type animals (Figure 3.2D). In contrast, *pdp-1* RNAi significantly reduced the mean and maximal lifespan of long-lived *daf-2(e1370)* and *age-1(hx546)* mutants (Figure 3.2B and 3.2C). We did not observe any effect of *pdhk-2* RNAi on the lifespan of wildtype and *daf-2* animals (Addendum). To examine the effect of increased dosage of *pdp-1*, we generated translational fusion strains of *pdp-1* fused to *gfp* driven by its own promoter (*pdp-1::gfp).* In addition, we also crossed *pdp-1::gfp* to *daf-2(e1370)* mutants to generate the *daf-2(e1370); pdp-1::gfp* strain. Overexpression of *pdp-1* results in a significant extension in lifespan compared to wild type worms (Figure 3.2D). Interestingly, *pdp-1* overexpression further extends the lifespan of *daf-2(e1370)* mutants (Figure 3.2B). Therefore, dosage modulation of PDP-1 regulates lifespan. We next asked if PDP-1 modulated

additional outputs of the IIS signaling pathway. We first tested whether PDP-1 regulates stress resistance by assaying the survival of *pdp-1* mutants and transgenic animals when exposed to heat stress at 37°C (Figure 3.2E). Dosage modulation of *pdp-1* affects the response to heat stress, with a *pdp-1* mutation decreasing and overexpression slightly enhancing thermotolerance (Figure 3.2E). Importantly the *pdp-1* mutation drastically reduced the thermotolerance of *daf-2* mutants. Besides longevity, dauer formation and the response to stress, the IIS pathway also regulates metabolism. Therefore, we next used Oil Red O Staining [44] and Sudan Black Staining [7] to investigate its role in regulating fat storage [45] (Figure 3.2F). *pdp-1* overexpression or mutation in a wild type background had negligible effects on fat storage. However, a *pdp-1* mutation drastically reduced the increased fat of *daf-2(e1370)* mutants (Figure 3.2F). This was observed in dauers, larval stage 3 (L3) animals and adults, suggesting that PDP-1 is an important regulator of fat storage in *daf-2* mutants. We did not observe any changes in fat storage when wild type and *daf-2* mutants were grown on E1α RNAi (Addendum). Therefore, PDP-1 modulates all four well-characterized outputs of the IIS pathway. *pdp-1(tm3734)* mutants exhibit a slow movement phenotype, which we quantified using locomotion assays (Figure 3). This slow movement was rescued by the *pdp-1::gfp* transgene. However, *pdp-1(tm3734); daf-2(e1370)* mutants also have an additional phenotype where the majority of the eggs laid remained unhatched. We quantified this phenotype with a brood size analysis of wild type worms, *daf-2(e1370), pdp-1(tm3734)* and *pdp-*

1(tm3734); daf-2(e1370) mutants (Figure 3.3). While *pdp-1(tm3734)* worms showed a slight decrease in the number of progeny compared to wild type and *daf-2* mutants, only 5% of the *pdp-1(tm3734); daf-2(e1370)* eggs yielded progeny (Figure 3.3). *daf-2* mutants have a slightly reduced brood size, and a *pdp-1* mutation severely enhances this phenotype [46,47]. Taken together, PDP-1 regulates multiple outputs of the IIS pathway as well as locomotion and brood size. These phenotypes are more severe, especially under conditions of reduced signaling as in the case of a *daf-2* or *age-1* mutant. Importantly, PDP-1 acts as a negative regulator of IIS, similar to DAF-18/PTEN.
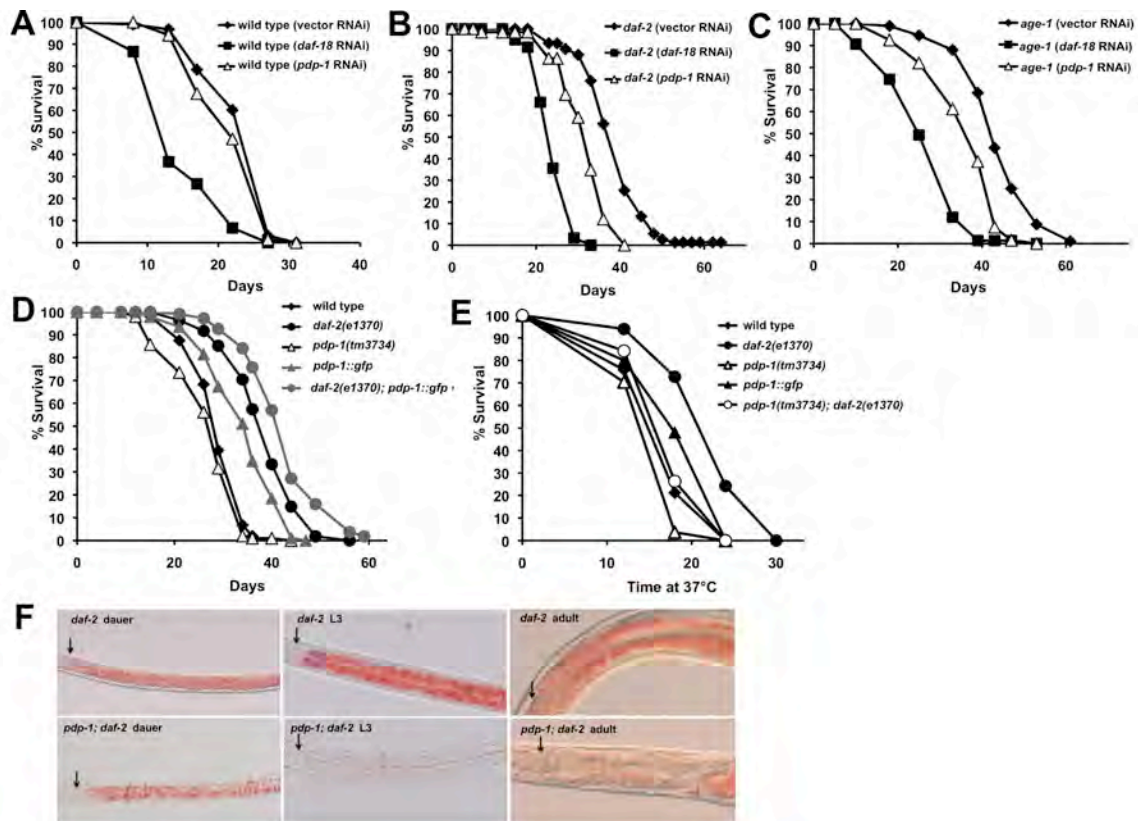
Figure 3.2: PDP-1 regulates multiple outputs of the IIS pathway

Data shown are from one representative experiment.

A) *pdp-1* RNAi does not significantly reduce the lifespan of wild type worms. Mean lifespan of wild type worms was 23.8 ± 0.5 days (n=93) on vector RNAi, 14.5 ± 0.9 days (n=34) on *daf-18* RNAi (p<0.0001) and 23.8 ± 0.5 days (n=93) and 22.6 ± 0.6 days (n=68) days on *pdp-1* RNAi (p<0.08).

B) The increased lifespan of *daf-2(e1370)* worms is reduced by *pdp-1* RNAi. Mean lifespan of *daf-2(e1370)* worms was 38.9 ± 0.9 days (n=75) on vector RNAi, 24.5 ± 0.5 days (n=59) on *daf-18* RNAi (p<0.0001) and 23.8 ± 0.5 days (n=93) and 31.7 ± 0.8 days (n=66) days on *pdp-1* RNAi (p<0.0001).

C) *pdp-1* RNAi reduces the increased lifespan of *age-1(hx546)* mutants. Mean lifespan of *daf-2(e1370)* worms was 42.8 ± 0.8 days (n=84) on vector RNAi, 28.0 ± 0.9 days (n=81) on *daf-18* RNAi (p<0.0001) and 36.5 ± 1.0 days (n=67) on *pdp-1* RNAi (p<0.0001).

D) *pdp-1* overexpression increases the lifespan of wild type and *daf-2(e1370)* worms while *pdp-1* mutants live slightly shorter than wild type animals. Mean lifespan of wild type worms was 29.4 ± 0.5 days (n=104), *pdp-1(tm3734)* mutants was 27.1 ± 0.7 days (n=98), p<0.05, *pdp-1::gfp* mutants was 34.5 ± 0.8 days (n=92) p<0.0001, *daf-2(e1370)* was 38.7 ± 0.7 days (n=108) and *daf-2(e1370); pdp-1::gfp* was 42.8 ± 0.7 days (n=105) days p<0.0001.

E) PDP-1 regulates thermotolerance. Mean survival of wild type worms was 18.3 ± 0.7 hours (n=37), *pdp-1(tm3734)* mutants was 17.1 ± 0.8 hours (n=27) p<0.2, *pdp-1::gfp* worms was 19.7 ± 0.9 days (n=25) p<0.09, *daf-2(e1370)* worms was 21.6 ± 0.6 hours (n=30) and *pdp-1(tm3734); daf-2(e1370)* worms was 18.6 ± 0.9 hours (n=19), p<0.0007.

F) Oil Red O staining reveals that *pdp-1(tm3734); daf-2(e1370)* worms store less fat than *daf-2* worms across different stages in the worm life cycle: dauers (left), L3 worms (middle) and adults (right). Arrows indicate the lower bulb of the pharynx.
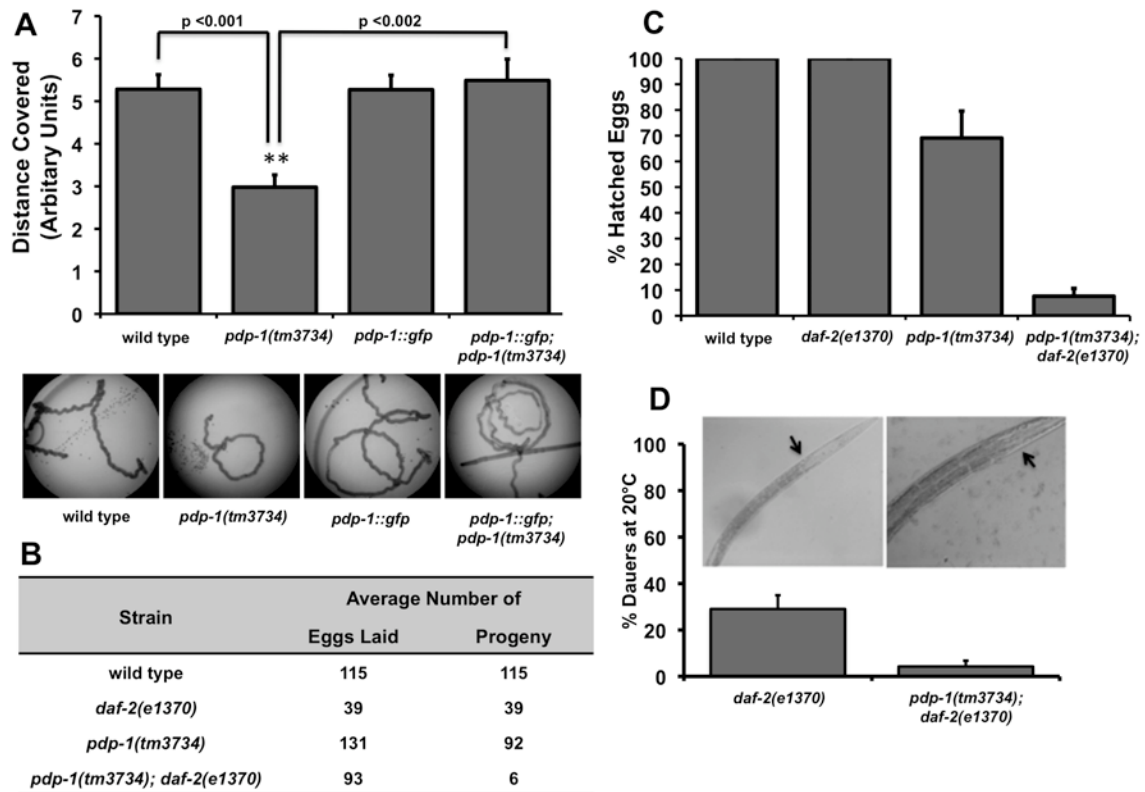
Figure 3.3: PDP-1 mutants have a slow movement phenotype and reduced brood size

Data shown are from one representative experiment. Error bars indicate the standard deviation among the different plates within one experiment.

A) *pdp-1(tm3734)* mutants have a slow movement phenotype when compared to wild type worms (p<0.001). This movement can be rescued by expression of a *pdp-1::gfp* transgene in the mutant background (p<0.002). Lower panel: Traces of wild type, *pdp-1(tm3734), pdp-1::gfp* and *pdp-1::gfp; pdp-1(tm3734)* worms moving on a lawn of OP50.

B) Brood size of wild type, *daf-2(e1370), pdp-1(tm3734)* and *pdp-1(tm3734); daf-2(e1370)* animals as scored after 22.5 hours (total number of eggs laid) and 38 hours (total number of progeny).

C) The % hatched eggs calculated from the number of progeny and number of eggs laid. *pdp-1(tm3734)* worms have fewer progeny (p<0.04) when compared to wild type worms, however, this phenotype is far more severe in *pdp-1(tm3734); daf-2(e1370)* worms (p<0.005).

D) A mutation in *pdp-1* suppresses *daf-2(e1370)* dauer formation, similar to the effect of *pdp-1* RNAi. (p<0.03).

3. PDP-1 positively regulates DAF-16

The FOXO transcription factor DAF-16 is a major target of the IIS pathway [2,48]. Under conditions of reduced IIS, DAF-16 translocates into the nucleus, where it regulates the expression of hundreds of direct as well as indirect target genes [12,13,49,50]. We therefore asked whether PDP-1 modulates DAF-16 subcellular localization as well as activity (Figure 3.4). *daf-2(e1370); daf-16::gfp* worms were grown on vector, *daf-18* and *pdp-1* RNAi, and DAF-16 nuclear/cytosolic localization was visualized using fluorescence microscopy. While DAF-16::GFP was mostly nuclear on vector RNAi, its localization was enriched in the cytosol on *pdp-1* RNAi, similar to *daf-18* RNAi throughout the body of the worm (Figure 3.4). The gene superoxide dismutase 3 *(sod-3)* is a direct DAF-16 target [11]. To test whether PDP-1 modulates transcriptional activity of DAF-16, we used a *Psod-3::gfp* reporter strain a *daf-2(e1370)* background [51]. *daf-2(e1370); Psod-3::gfp* worms were grown on vector, *pdp-1, daf-18* and *daf-16* RNAi and GFP expression was visualized using fluorescence microscopy and scored as low, medium or high (Figure 3.4). GFP expression was markedly lower on *pdp-1* RNAi compared to vector RNAi, suggesting that PDP-1 modulates DAF-16 transcriptional activity. To further validate these results, we used quantitative real-time PCR (Q-PCR) to look at the expression levels of well-known DAF-16 target genes [52] in *daf-2(e1370), pdp-1(tm3734); daf-2(e1370)* and *daf-16(mgDf50); daf-2(e1370)* worms. Notably, the expression of *sod-3, sod-5* and *hsp-12.6* were reduced in *pdp-1(tm3734); daf-2(e1370)*

mutants relative to *daf-2(e1370)*. Therefore PDP-1 positively regulates DAF-16 activity under conditions of reduced IIS.
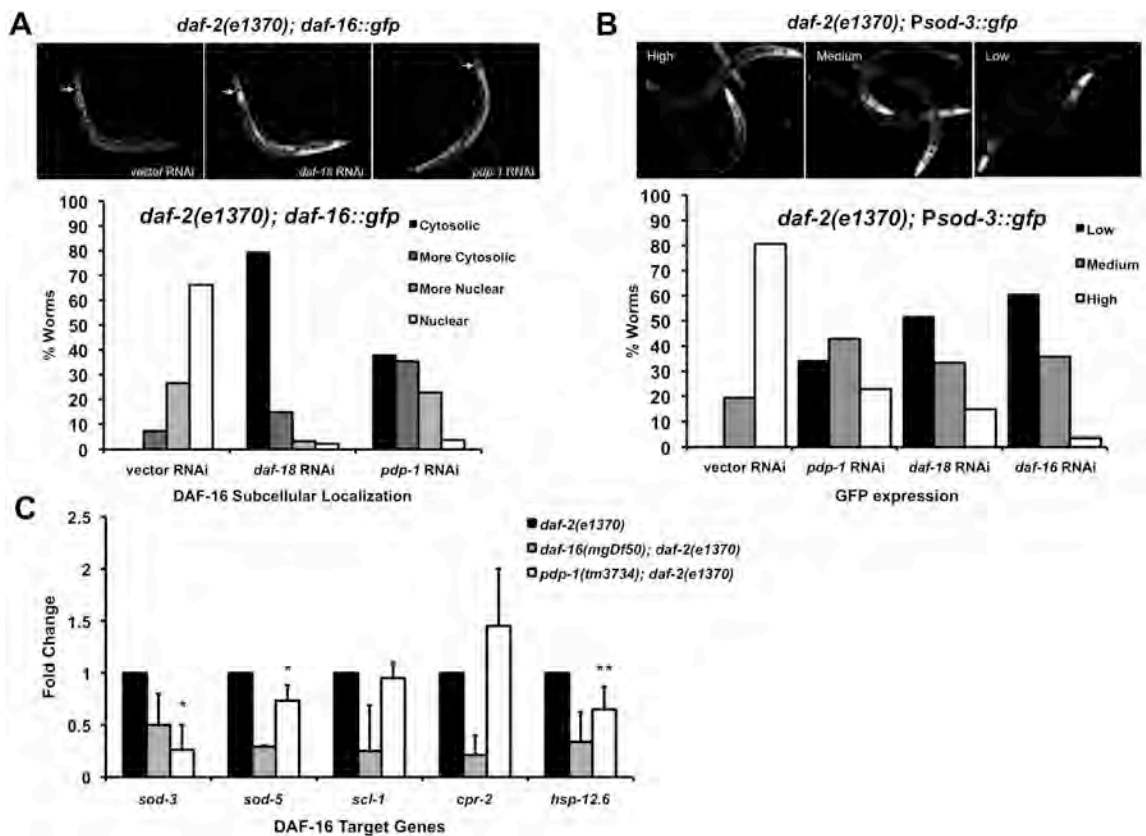
Figure 3.4: PDP-1 regulates DAF-16 nuclear localization and transcriptional activity

A) DAF-16::GFP localization visualized in *daf-2(e1370); daf-16::gfp* worms on vector, *daf-18* and *pdp-1* RNAi (top panel) and quantification of DAF-16::GFP nuclear-cytosolic localization (lower panel). Data shown are from one representative experiment. (n=68 on vector RNAi, n=88 on *daf-18* RNAi and n=79 on *pdp-1* RNAi).

B) Representative images of high, medium and low GFP expression in *daf-2(e1370); Psod-3::gfp* worms (top panel). Quantification of GFP expression in *daf-2(e1370);Psod-3::gfp* worms on vector, *daf-18, pdp-1* and *daf-16* RNAi

(Lower panel). Data shown are from one representative experiment (n= 31 on vector RNAi, n=35 on *pdp-1* RNAi, n=27 on *daf-18* RNAi and n=28 on *daf-16* RNAi).

C) Levels of known DAF-16 targets are reduced in *pdp-1(tm3734); daf-2(e1370)* worms when compared to *daf-2(e1370)* worms. Data shown is an average of three independent repeats.

4. PDP-1 acts in the DAF-7/ TGF-β signalling pathway

Thus far our data indicates that PDP-1 regulates multiple outputs of IIS as well as DAF-16 activity. Using dauer formation as the readout, we performed genetic epistasis experiments to identify PDP-1's substrate. Our results ruled out the role of PDH in regulating the dauer phenotype. However, it was likely that pathways that were parallel or intersecting with the IIS pathway could also affect dauer formation. We first focused mutants of genes within the IIS pathway downstream of *daf-2* (Table 3.1). *pdk-1(sa680), daf-2(e1370); akt-1(ok525)* and *daf-2(e1370); akt-2(ok393)* mutants were maintained on vector, *daf-18* and *pdp-1* RNAi and dauer formation was assayed at the appropriate temperatures. Interestingly, *pdp-1* RNAi resulted in suppression of dauer formation of *pdk-1(sa680)* mutants*, daf-2(e1370); akt-1(ok525)* and *daf-2(e1370); akt-2(ok393)* worms, suggesting that PDP-1 functioned independent of components of the IIS pathway (Table 3.1).

Dauer formation in *C.elegans* is also regulated by a TGF-β like pathway, besides the IIS pathway [19,20,21]. In this pathway the DAF-7 BMP-like ligand activates the DAF-1/DAF-4, the Type I/II receptors, which phosphorylate the R-SMAD proteins DAF-8 and DAF-14. DAF-8 and DAF-14 antagonize the transcriptional activity of the co-SMAD DAF-3 Co-SMAD and the Ski repressor DAF-5 and promote reproductive growth [22,23,24,25,26,27]. Mutations *daf-7, daf-1, daf-4, daf-8* and *daf-14* result in constitutive dauer-formation and mutations in *daf-3* and/or *daf-5* suppressing these phenotypes [21,28]. Since *pdp-1* RNAi resulted in suppression of dauer formation of the IIS pathway mutants, we

therefore performed genetic epistasis assays with mutants of the TGF-β pathway. In these assays, TGF-β pathway mutants were maintained on vector RNAi, *pdp-1* RNAi and *daf*-3 RNAi (as a positive control; Table 2). Dauer formation of *daf-7(e1372)* mutants was suppressed on *pdp-1* RNAi similar to, *daf-3* RNAi, suggesting that *pdp-1* acts downstream of *daf-7* (Table 3.2).

The R-SMAD proteins, DAF-8 and DAF-14 proteins contain a conserved SSXS phosphorylation motif that has been shown to be important for R-SMAD activation in mammals [27,53,54]. Upon activation, R-SMADs can associate with a Co-SMAD to regulate the transcription of hundreds of genes [55]. While both DAF-8 and DAF-14 are thought to function redundantly, *daf-8* mutants exhibit a much weaker constitutive dauer formation phenotype compared to *daf-14* mutants, suggesting that DAF-14 is more important of the two R-SMADs for dauer formation [27,53]. However, expressing *daf-8* in a *daf-14* mutant rescues the constitutive dauer phenotype [27]. We tested dauer formation of *daf-14(m77)* mutants on vector, *pdp-1* and *daf-3* RNAi. Interestingly, *pdp-1* RNAi had no effect on *daf-14* dauer formation. We next looked at dauer formation of *daf-8(m85)* mutants and again observed that *pdp-1* RNAi had no effect, while in both cases, *daf-3* RNAi resulted in dauer suppression (Table 3.2), similar to *daf-5* RNAi (data not shown). These results indicate that *pdp-1* acts at the level of the R-SMADS *daf-14* and *daf-8.* To confirm these results, we investigated whether *pdp-1* RNAi could suppress dauer formation of *daf-2(e1370); daf-3(mgDf90)* double mutants. In this strain, input from the TGF-β pathway is removed due to

the *daf-3* null mutation, and dauer formation is presumably mediated through activated DAF-16 [37] Therefore, if *pdp-1* was indeed acting in the TGF-β pathway, we would not see any effect of *pdp-1* RNAi on *daf-2(e1370); daf-3(mgDf90)* double mutants. Conversely, if *pdp-1* acted specifically through the IIS pathway, we would expect *pdp-1* RNAi to suppress dauer formation in this strain. Expectedly, we found that, *pdp-1* RNAi had no effect on *daf-2(e1370); daf-3(mgDf90)* double mutants (Table 3.2). DAF-3 itself is unlikely to be a substrate for PDP-1, as similar to mammalian Co-SMADs, it lacks the C-terminal phosphorylation motif [24]. Therefore, *pdp-1* acts in the TGF-β pathway at the level of the R-SMADs *daf-8* and *daf-14.*

Table 3.1: Genetic Epistasis Analysis using IIS Mutants

Strain	% Dauers ± Std. Dev (n)		
	vector RNAi	*daf-18* RNAi	*pdp-1* RNAi
pdk-1(sa680)[a,b]	85.0 ± 4.7 (520)	-	35.3 ± 2.5 (327)*
daf-2(e1370)[c]	8.3 ± 8.6 (476)	0 (331)	5.5 ± 1.0 (241)
daf-2(e1370); akt-1(ok595)[c]	36.9 ± 1.4 (390)	3.5 ± 0.9 (265)*	16.0 ± 0.4 (375)*
daf-2(e1370)[d]	75.6 ± 4.8 (247)	0.3 ± 1.0 (777)*	17.3 ± 8.2 (597)**
daf-2(e1370); akt-2(ok393)[d]	61.1 ± 15.3 (289)	4.1 ± 1.7 (308)**	11.5 ± 3.6 (301)**

[a] The assays were performed at 25°C

[b] As previously reported, *pdk-1(sa680)* mutants survive poorly on *daf-18* RNAi.

[c] The assays were performed at 19.2°C

[d] The assays were performed at 20°C

* $p < 0.01$
** $p < 0.05$

Table 3.2: Genetic Epistasis Analysis using TGF-β signaling Mutants

Strain	% Dauers ± Std. Dev (n)		
	vector RNAi	daf-3 RNAi	pdp-1 RNAi
daf-7(e1372)[a]	85.3 ± 1.1 (612)	43.4 ± 0.8 (134)*	32.2 ± 4.9 (122)*
daf-14(m77)[b]	81.7 ± 5.6 (543)	18.1 ± 8.9 (441)**	88.7 ± 1.3 (535)
daf-8(m85)[a]	32.0 ± 9.7 (392)	2.3 ± 1.8 (396)**	34.6 ± 9.1 (430)
daf-2(e1370); daf-3(mgDf90)[c]	50.8 ± 0.4 (302)	-	49.5 ± 2.5 (270)

[a] The assays were performed at 22.5°C

[b] The assays were performed at 20°C

[c] The assays were performed at 19.2°C

* $p < 0.01$

**$p < 0.05$

5. Insulins are a possible connection between TGF-β signaling and IIS

How can these two pathways, hitherto considered parallel to each other, be mechanistically linked? Thus far our data suggests that PDP-1, a component of the TGF-β pathway can modulate multiple phenotypes of the IIS pathway and positively regulate DAF-16. A feed-forward model that has been proposed to connect TGF-β signaling to the IIS pathway suggests insulins as a possible link [56,57]. The *C.elegans* genome encodes 40 insulin genes [58,59] (WormBase 215: www.wormbase.org). Studies using mutants and RNAi have characterized some of the insulins as agonists or antagonists of the IIS pathway [13,58,59,60]. Importantly, microarray studies have identified several insulin genes that are regulated by the TGF-β pathway, including *ins-1, ins-4, ins-5, ins-6, ins-7, ins-17, ins-18, ins-30, ins-33*, *ins-35* and *daf-28* [56,61]. We tested changes in the levels of these insulins in TGF-β pathway mutants using Q-PCR (Figure 3.5B, Table 3.3 & 3.4). Interestingly, both *pdp-1(tm3734)* and *daf-3(mgDf90)* showed elevated levels of several insulins as compared to wild type worms (Figure 3.5A). In contrast expression of these insulins was markedly reduced in *daf-14(m77)* mutants (Figure 3.5B). In addition, the gene *mdl-1* has been previously described as a DAF-3 target, with DAF-3 suppressing *mdl-1* transcript levels [62]. Consistent with this, we find that *pdp-1* RNAi results in increased *mdl-1* expression in a *Pmdl-1::gfp* transgene (Figure 3.5E). Therefore, similar to DAF-3, PDP-1 acts as a negative regulator of the DAF-7 pathway.

Next, we tested changes in insulin gene expression in *daf-2(e1370)* mutants, and compared the results with *pdp-1(tm3734); daf-2(e1370)* and *daf-16(mgDf50); daf-2(e1370)* double mutants. Several insulins were changed relative to *daf-2(e1370)* worms, however the trend between *pdp-1(tm3734); daf-2(e1370)* and *daf16(mgDf50); daf-2(e1370)* was not always similar (Figure 3.5C). Notably, *ins-1* levels were drastically reduced in *pdp-1(tm3734); daf-2(e1370)* worms relative to *daf-2(e1370)* worms. *ins-1* has been characterized as a potential antagonist of the IIS pathway [58]. Interestingly, *ins-7* levels were elevated both double mutants (Figure 3.5D,E). Previous studies have shown *ins-7* to be an agonist of the IIS pathway as well as a DAF-16 target gene [13,63]. We did not observe any changes in *ins-18,* another potential DAF-16 target [13]. In addition, we did not detect any appreciable differences in insulin gene expression in *daf-16(mgDf50)* mutants. We were unable to detect *ins-33* and *ins-35* transcripts in all the strains tested. Our results suggest that PDP-1 modulates TGF-β signaling to regulate expression of several insulins that can potentially feed into or antagonize the IIS pathway to regulate DAF-16 and its associated phenotypes.

Table 3.3: List of insulins tested

Insulin	Lifespan or Dauer Phenotype in	
	Mutants/RNAi	Overexpression

* Wormbase (WS204) www.wormbase.org
nt: not tested
a Pierce et. al, Genes & Dev, 2001
b Li et. al, Genes & Dev, 2003
c Kao et. al, Cell, 2007
d Murphy et. al, Nature 2003
e Murphy et. al, PNAS 2007
f Kawano et. al, Biochem Biophys Res Commun, 2000
g Ouellet et.al, Development, 2008
h McElwee et. al, Aging Cell, 2003
i Hristova et. al, Mol Cell Biol, 2005
j Malone et. al, Genetics, 1994
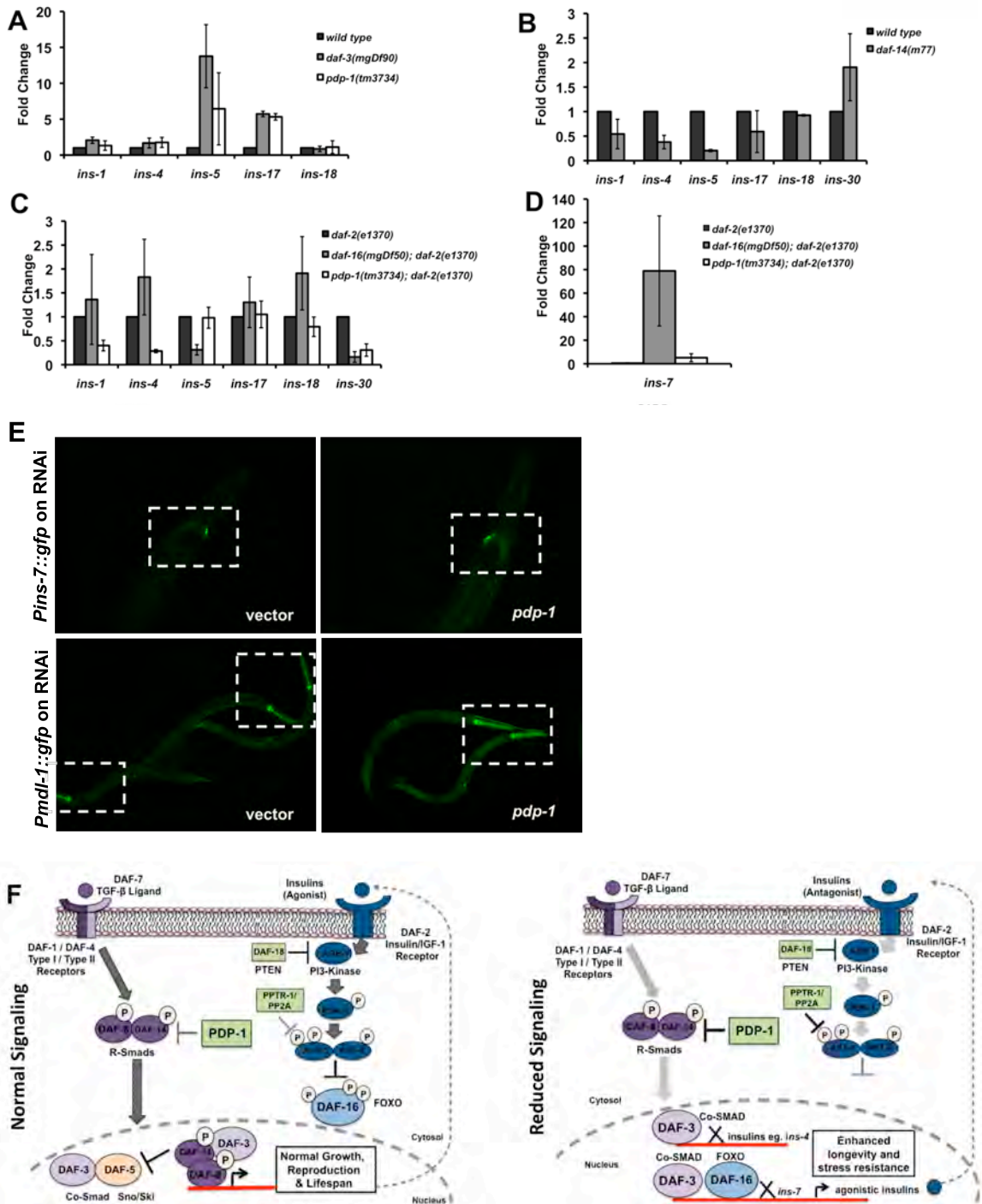
Figure 3.5: PDP-1 modulates the expression of insulin genes that possibly feed into the IIS pathway. Data shown are an average of at least two independent repeats.

A) The expression of several insulins is elevated in both *pdp-1(tm3734)* and *daf-3(mgDf90)* mutants

B) The same insulins show decreased expression on *daf-14(m77)* mutants

C) Insulin levels are changed in *pdp-1(tm3734); daf-2(e1370)* mutants compared to *daf-2(e1370)*

D) *ins-7* levels are drastically elevated in *daf-16(mgDf50); daf-2(e1370)* and *pdp-1(tm3734); daf-2(e1370)* worms as compared to the parental *daf-2(e1370)* strain

E) *ins-7* expression is increased upon *pdp-1* RNAi (top panel, boxes show the lower pharyngeal bulb). PDP-1 negatively regulates the expression of the DAF-3 target *mdl-1,* similar to DAF-3 (lower panel, boxes show the pharynx)

F) Model to link the IIS and TGF-β signaling pathways: Under normal signaling conditions, the TGF-β signaling pathway regulates the expression of insulins that activate the IIS pathway, resulting in the negative regulation of DAF-16. Under reduced signaling conditions, PDP-1 further downregulates signals through the TGF-β pathway to promote DAF-3 mediated repression of insulin genes. As a consequence, reduced IIS results in the enhancement of DAF-16 regulated phenotypes such as longevity, dauer formation and stress resistance.

Table 3.4: Summary of trends observed in the Q-PCR Experiments

Insulin	Trend relative to wild type worms[a]			Trend relative to *daf-2(e1370)* worms		
	daf-3(mgDf90)	*pdp-1(tm3734)*	*daf-14(m77)*	*daf-16(mgDf50); daf-2(e1370)*	*pdp-1(tm3734); daf-2(e1370)*	*daf-2(e1370); daf-3(mgDf90)*
ins-1	increased	increased	decreased	no change	decreased	decreased
ins-4	increased	increased	decreased	no change	no change	no change
ins-5	increased	increased	decreased	decreased	decreased	nd
ins-7*	nd	*	*	increased	increased	nd
ins-17	increased	increased	decreased	no change	no change	no change
ins-18	no change	no change	no change	no change	no change	no change
ins-30[b]	variable	variable	no change	decreased	decreased	nd
ins-33	nd	nd	nd	nd	nd	nd
ins-35	nd	nd	nd	nd	nd	nd
daf-28[b]	nd	variable	variable	decreased	variable	nd

nd – No amplicon detected in multiple experiments
ins-7 levels were similar in *daf-14* and *pdp-1* mutants
a None of the insulins tested showed changes in *daf-16(mgDf50)* mutants
b *ins-30* and *daf-28* levels showed opposite trends in multiple repeats

Discussion

We identified PDP-1 from an RNAi screen for serine/threonine phosphatases that modulate *daf-2* dauer formation. *C.elegans* PDP-1 is homologous to mammalian pyruvate dehydrogenase phophatase, a metabolic enzyme that is a positive regulator of the pyruvate dehydrogenase enzyme complex (PDHc). In humans, mutations in the E1 subunit of PDH cause Leigh's disease, neurological disorder where patients suffer from lactic acidosis [83,84]. Mutations in PDP manifest in the form of symptoms similar to Leigh's disease [85,86,87]. In worms, *pdp-1* mutants have significantly elevated levels of lactate relative to wild type worms (Addendum). This however, is unlikely to account for the suppression of dauer formation by *pdp-1* RNAi or mutation, as *daf-2* mutants themselves have much higher lactate levels than wild type worms (Addendum). Remarkably, RNAi other components of the PDHc in *C.elegans* do not affect *daf-2* dauer formation, even though we observed a significant reduction in transcript levels. Microarray and SAGE studies on dauers have indicated that genes involved in anaerobic metabolism are upregulated while genes involved in the TCA cycle and mitochondrial oxidative phosphorylation are downregulated, suggesting that PDHc activity may not be critical for dauer diapause [1,2,3]. In particular, RNAi of E1α subunit did not affect fat storage or lifespan either. In mammals, the modulation of PDHc is tightly coupled to IIS, with the pyruvate dehydrogenase kinase being upregulated under conditions of starvation as a direct transcriptional target of FOXO [88]. We found that in worms, *pdhk-2* RNAi resulted in allele-

specific suppression of *daf-2* dauer formation. It will be interesting to further explore the role of *pdhk-2* in modulating the IIS pathway.

Further, annotations indicate that the *C. elegans* genome encodes approximately 60 serine/threonine phosphatases, in contrast to the 400 plus protein kinases, suggesting that phosphatases are likely to have a number of cellular substrates [38,67]. PDP-1 is broadly expressed in the cell, and we observe expression in the cytosol, nucleus and membrane fractions (Addendum). Besides dauer formation, we find that PDP-1 also regulates longevity, fat storage and stress resistance. Interestingly, these phenotypes are more severe in mutants such as *daf-2* and *age-1,* where IIS is reduced. Consistent with this, we find that PDP-1 positively regulates DAF-16 activity. A mutation in *pdp-1* had only a slight reduction in the different outputs of IIS in wildtype worms. Perhaps PDP-1's function is more important under conditions of stress or low food availability, when DAF-16 activation is required. Indeed, mutations in *daf-16* only slightly shorten lifespan and stress resistance and have little to no effect on fat storage. However, in a *daf-2* or *age-1* background, loss of *daf-16* results in dramatic effects on the various phenotypes regulated by the IIS pathway.

Intriguingly, genetic epistasis analyses place PDP-1 in the DAF-7/TGF-β pathway, at the level of the R-SMAD proteins DAF-14 and DAF-8. A recent functional RNAi screen for serine/threonine phosphatases that modulate BMP signaling identified PDP as a SMAD1 phosphatase in *Drosophila* S2 cells and mammalian 293T cells [68]. Our study complements these findings and reveals

a molecular conservation in the role of PDP-1 in regulating TGF-β signaling. Early genetic epistasis studies had suggested that TGF-β signaling and IIS pathways are parallel signaling pathways that modulate dauer diapause [29]. Importantly, in these studies, the conclusion was that both these pathways acted independently, and it was the IIS pathway that regulated longevity and stress resistance [29,30]. However, more recent studies have suggested that TGF-β pathway regulates the expression of insulins, and this results in a feed-forward model, where signals from the TGF-β pathway are relayed to modulate activity of the IIS pathway as well as DAF-16 (Figure 3.5) [56,61]. In support of this model, we find TGF-β signaling regulates the expression of several insulin genes. The expression of many insulins were markedly elevated in *pdp-1* as well as *daf-3* mutants, suggesting that both genes normally act to negatively regulate their expression. This is in agreement with previous studies that identify DAF-3 as a repressor of gene expression [62,69]. The R-SMADs DAF-8 and DAF-14 antagonize DAF-3 activity, and consistent with this, *daf-14* mutants showed reduced expression of several insulins. For example, INS-4 has been previously reported as a positive regulator TGF-β pathway and a suppressor of dauer formation of *daf-7* and *daf-8* mutants [70]. *ins-4* transcript levels were elevated in *pdp-1* and *daf-3* mutants but reduced in *daf-14*. Supporting these observations, a recent mammalian study has identified the insulin gene as a direct target of TGF-β signaling [71].

Interestingly, the majority of the insulins tested were unchanged in *daf-16* mutants. An exception was *ins-7*, which was increased in *daf-16(mgDf50); daf-2(e1370), pdp-1(tm3734)* as well as *pdp-1(tm3734); daf-2(e1370)* mutants. Previous studies using *ins-7* mutants and RNAi have identified this insulin as an agonist of the IIS pathway and a negative regulator of lifespan as well as dauer formation [13,63]. In contrast, levels of *ins-1,* which has been identified as an antagonist of the DAF-2 pathway [58], were reduced in *pdp-1(tm3734); daf-2 (e1370).* Therefore, in the absence of PDP-1, increased levels of agonists such as INS-7 or reduced levels of antagonists such as INS-1 may hyperactivate the DAF-2 pathway to negatively regulate DAF-16, thereby affecting the enhanced lifespan, stress resistance, dauer formation and fat storage of *daf-2* mutants.

A clear interpretation of our results is complicated by two factors. First, the sheer number of insulins in the worm makes it difficult to assess whether they are functionally distinct. Secondly, the role of temperature in modulating the readouts of the pathway has not been closely explored. For example, we observe the effects of *pdp-1* RNAi on *daf-2* lifespan at 15°C but the effect decreases at a higher temperature, as the pathway gets more inactive. It is therefore likely that a certain level of signaling through the pathway is required to activate and target PDP-1 to its substrates. At higher temperatures such as 20°C or 25°C, there may be extremely low levels of phosphorylated substrate available for PDP-1. Therefore, temperature and the level of signaling are important additional inputs that need to be considered to better understand the crosstalk between the IIS

and the TGF-β pathways. Our results suggest a model where under normal (favorable) signaling conditions, signals through the TGF-β pathway result in the activation of the SMAD transcriptional complex, that regulates the expression of insulins that activate the IIS pathway to favor growth, reproduction and normal lifespan (Figure 3.5E). However, under reduced signaling conditions, presumably when food is limiting or under harsh survival conditions, TGF-β signaling is further downregulated by PDP-1 to activate the co-SMAD DAF-3, to regulate the repression of insulin genes that may feed into the IIS pathway. Recent studies in worms indicate that when active, DAF-3 can further downregulate the TGF-β pathway to suppress gene expression of the *daf-7* ligand and *daf-8* R-SMAD [27].

In conclusion, PDP-1 acts via the DAF-7/TGF-β pathway to negatively regulate IIS and promote DAF-16 nuclear localization as well as activity. PDP-1 may mediate this function in part by negatively regulating TGF-β signaling to repress expression of several insulins that feed into the IIS pathway. In humans, dysregulation of TGF-β signaling and the insulin/IGF-1 signaling axis have been implicated in the onset of age-associated diseases such as type 2 diabetes and cancer [72,73,74,75,76]. Future studies exploring the interactions between these two pathways as well as the factors that modulate these interactions may ultimately provide a better understanding of the pathophysiology of these diseases.

Materials and Methods

Strains

All strains were maintained at 15°C using standard *C. elegans* techniques [77]. For all RNAi assays, worms were maintained on the RNAi bacteria for two generations except for the assays on the PDHc RNAi. Strains used in this manuscript are listed in Table 3.5.

RNAi based assays

RNAi plates were prepared as previously described [37]. L4 worms were picked onto fresh RNAi plates and maintained for two generations prior to the assay, with the exception PDHc RNAi plates. Worms exhibit lethality when maintained on the following RNAi clones: T05H10.6 (E1α), C04C3.3 (E1β), F23B12.5 (E2), or LLC1.3 (E3) [78]. To circumvent this problem, strains were maintained on vector RNAi for two generations and transferred to E1α, E1β, E2 or E3 plates prior to the assay.

Strain Construction

For the *pdp-1(tm3734);daf-2(e1370)* double mutant, *daf-2(e1370)* males were mated to *pdp-1(tm3734)* hermaphrodites. A total of 30 F1 progeny were picked onto individual plates and allowed to have progeny at 25 °C. From the F2 progeny on each plate, dauers were selected and transferred to fresh plates and incubated for an additional 24 hours at 25 °C. The next day, the dauers were

allowed to recover at 15 °C until they reached adulthood. Subsequently, adult worms were picked onto individual plates and transferred to 25 °C and allowed to have progeny. Among the F3 progeny, we observed that some plates had 100% dauers at 25°C, while worms in some of the plates exhibited a developmental delay and could not form complete dauers even after 5-6 days at 25°C. Worms from both sets of plates were recovered, picked to individual plates and allowed to self at 15°C. Parents were then tested for *pdp-1(tm3734)* deletion by PCR. As anticipated, the *pdp-1(tm3734);daf-2(e1370)* double mutants are unable to form 100% dauers at 25°C.

The *daf-2(e1370);pdp-1::gfp* strain was made by crossing *daf-2(e1370)* males to either *pdp-1::gfp* hermaphrodites. About 30 F1 animals were transferred to individual plates and allowed to have progeny at 25°C. From the progeny, F2 dauers were selected from each plate and allowed to recover at 15°C. The recovered adult worms were then checked for the presence of GFP, and GFP-positive worms were transferred to individual plates and incubated at 25 °C. Plates where 100% of the progeny were dauers and GFP positive were selected and established as the strain for the assays.

Dauer assays

Strains were maintained on RNAi plates for two generations or regular OP50 plates at 15°C. Dauer assays were performed by picking approximately 100 eggs onto 2 fresh plates and incubated at the appropriate temperature. The *pdk-*

1(sa680), daf-7(e1372) and *daf-14(m77)* worms have a strong Egl phenotype. For dauer assays on these strains, gravid adult worms growing on the RNAi plates were washed off the plate with sterile PBS onto a 1.5 mL eppendorf tube. After 2 washes at 2000g for 30 seconds, the adults were vortexed for 5 mins in 5ml of 1N sodium hydroxide and 3% sodium hypochlorite (final concentration). The samples were then washed twice with sterile PBS and eggs were aspirated with a glass pipette onto fresh RNAi plates. For all dauer assays, plates were scored for the presence of dauers or non-dauers after 3.5-5.5 days, depending upon the strain. Dauer assays were performed at the temperature indicated.

Lifespan Assays

Strains were maintained at 15ºC and synchronized by picking eggs onto fresh RNAi or OP50 plates. Approximately 60 young adult worms were transferred per plate to a total of three fresh RNAi or regular OP50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration of 0.1 mg/ml [79]. All RNAi-based lifespan assays were carried out at 15ºC. Lifespans on OP50 plates were performed at the temperature indicated. Survival was scored by tapping with a platinum wire every 2-3 days. Worms that died from vulval bursting were censored from the analysis. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

Heat Stress assay

Strains were maintained on RNAi or regular OP50 bacteria at 15⁰C, as described above. From these plates, approximately 30 young adult worms were picked onto fresh RNAi or regular plates and upshifted to 20⁰C for 6 hrs. The plates were then transferred to 37°C and heat stress-induced mortality was determined every few hours till all the animals died.

Fat staining

Strains maintained RNAi on regular OP50 plates were synchronized by picking eggs on to fresh plates and grown synchronously at 15⁰C. The plates were then upshifted to 20⁰C for 8 hours, at the L2 stage to get L3 worms and at the L4 stage to get young adult worms. The worms were then washed off the plates into microcentrifuge tubes and incubated in 1x PBS buffer for 20 minutes on a shaker at RT. After 2 washes at 3000rpm for 30 seconds with 1x PBS, the strains were fixed according to the type of staining performed. Oil Red O and Sudan black staining was performed as previously described [44] [37] [45]. After incubation overnight at RT, worms were mounted on slides and visualized using the Zeiss Axioscope 2+ microscope.

Quantification of Fat Staining

For Sudan Black Staining, we used Image J software to measure the average pixel intensity for a 84-pixel radius below the pharynx of each animal in the anterior intestine area.   Next, an 84-pixel radius of the background was

measured, and subtracted from the values obtained for the staining. At least 10 animals were measured for each RNAi clone. Significance was determined by Student's t-test

For Oil Red O Staining, Image J was used to separate out each color image into its RGB channel components. As previously described [45], Oil Red O absorbs light at 510 nm and therefore, the green channel was used for further analysis. We measured the average pixel intensity for a 84-pixel radius below the pharynx of each animal in the anterior pharynx area. We next measured a 84-pixel radius of the background, which was later subtracted from the values obtained from the staining. At least 10 animals was measured for each RNAi clone. Significance was determined by Student's t-test.

DAF-16::GFP localization assay

DAF-16 localization assays were performed as previously described [38,52]. *daf-2(e1370); daf-16::gfp* worms were maintained on RNAi plates at 15ºC similar to the dauer assays. Approximately 30 L4 worms were transferred to fresh RNAi bacteria and the plates were shifted to 20ºC for 1hr. The worms were visualized under a fluorescence microscope (Zeiss Axioscope 2+ microscope). Worms were classified into four categories based on the extent of DAF-16::GFP nuclear-cytoplasmic distribution: completely cytosolic, more cytosolic than nuclear in most tissues, more nuclear than cytosolic in most tissues and completely nuclear.

***Psod-3::gfp* expression**

Quantification of *Psod-3::gfp* was performed as previously described [38]. *daf-2(e1370);sod-3::gfp* worms were grown at 15⁰C on RNAi as described above. Approximately 30 L4 animals were transferred to fresh RNAi bacteria and shifted to 25°C for 1 hr. The expression of *sod-3::gfp* was visualized using Zeiss Axioscope 2+ microscope. GFP expression was categorized as follows:

High: GFP expression seen throughout the worm

Medium: Weak expression detected in the body of the worm along with the head and the tail

Low: Low GFP expression only detected in the head and tail

Transgenic worms

Promoter and ORF entry clones of *pdp-1* obtained from the promoterome and ORFeome were combined using multisite Gateway cloning (Invitrogen) into the pDEST-DD03 or the R4-R2 GFP destination vectors to create the *Ppdp-1::gfp* or *Ppdp-1::pdp-1^ORF^::gfp* constructs. [80,81] All constructs contain the *unc-119* minigene. The vectors were verified by sequencing as well as restriction digestion. Transgenic worms were generated by ballistic transformation into *unc-119(ed3)* mutant worms as previously reported (Biorad, USA)[80]. Integrated lines that were obtained were used for further analyses. For the *pdp-1::gfp* translational fusion strain, additional integrated lines were obtained my

integration of extrachromosomal array lines by UV irradiation as previously described [82]. All translational fusion lines were backcrossed 4x to wild type before further analysis.

RT-PCR experiments

For all RT-PCR experiments, strains were maintained at 15ºC. Eggs were obtained from gravid adult worms by hypochlorite treatment described earlier. The eggs were seeded onto large plates maintained at 15ºC until the worms entered the L4 stage. The plates were then upshifted to 20ºC for 8 hours until they became young adults. Worms were then collected with sterile 1xPBS and washed twice at 2000g for 30 seconds. The supernatant was removed, and 0.5 ml of AE buffer (50 mM acetic acid, 10 mM EDTA), 0.1 ml of 10% SDS, and 0.5 ml of phenol was added to the worm pellet and the mixture was vortexed vigorously for 1 min, followed by incubation at 65°C for 4 min. Total RNA was purified by phenol:chloroform extraction and ethanol precipitation. The quality of the RNA isolated was determined by checking the 28 S and 18 S RNA on an agarose gel. 2 ug of total RNA was used for making cDNA using the SuperScript cDNA synthesis kit (Invitrogen, USA). The expression of the DAF-16 target and insulin genes was checked by RT-PCR using the SYBR® Green PCR Master Mix and 7000 Real-Time PCR System (Applied Biosystems, USA). The relative expression of the genes tested was compared to actin as an internal loading control.

Locomotion Assay

Young adult wild type and *pdp-1(tm3734)* worms were picked onto 6 individual plates each. After 5 minutes, the worms were picked off the plate. The average distance covered was calculated by measuring the traces on the bacterial lawn using NIH ImageJ.

Brood Size Measurements

Wild type, *daf-2(e1370), pdp-1(tm3734)* and *pdp-1(tm3734); daf-2(e1370)* worms were maintained at 15ºC. 5 L4 worms were picked onto individual plates and allowed to lay eggs at 22.5ºC. Worms were transferred to a new plate every 12 hours. After 22.5 hours, the parental worms were picked off the plates, and the total number of eggs laid was scored. The number of progeny from these eggs was scored again after 38 hours. The % hatched eggs was calculated as a percentage of the average number of progeny over the average number of eggs laid.

Lactate Assay

Strains were maintained at 15ºC until L4s and transferred to 20ºC until they became young adults. Worms were washed twice with PBS and frozen at -80ºC. For the lactate assay, worms were sonicated in the lactate assay buffer using a Misonix (3000) sonicator (Misonix, USA; power output set at 4, 2 pulses of 10 secs each with 1 min interval between pulses). The lysate was clarified by

centrigufation at 12000 rcf for 5 minutes and protein content from the lysate was estimated by Quick Bradford (Pierce). The lysate was then deproteinized using a 10kDa filter (Biovision) by centrifugation at 6000 rcf for 40 minutes. The samples were then assayed in triplicates according to the manufacturer's instructions (Biovision Lactate Assay Kit II).

Fractionation Experiments

Wild type, *pdp-1::gfp* and *myo-3::gfp* worms were grown at 20 degrees and washed twice with 1 x PBS. Worms were sonicated using a Misonix (3000) sonicator (Misonix, USA; power output set at 4, 3 pulses of 10 secs each with 1 min interval between pulses) in the specific buffers provided by the manufacturer (Qiagen QProteome Cell Fractionation Kit). Fractionation was carried out according to the manufacturer's instructions and protein content from the lysate estimated by Quick Bradford (Pierce). 50ug of protein lysate was used for western blot analysis. Protein samples were resolved on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST (Tris Buffered Saline containing 0.05% Tween 20, ph 7.4) containing 5% non-fat milk at RT for 1 hour. Membranes were then overnight with 1:1000 dilutions of antibodies in 5% non-fat milk at 4 ºC. Membranes were washed 3 times with TBST and then incubated win 5% non-fat milk containing a 1:10,000 dilution of the secondary antibody.

Table 3.5: List of strains used in this study

	Strain	Strain #	Additional information
1.	wild type (N2)		
2.	*daf-2(e1370)*	CB1370	
3.	*daf-2(e1368)*	DR1572	
4.	*pdp-1(tm3734)*	HT1666	Backcrossed to N2 4x
5.	*pdp-1(tm3734); daf-2(e1370)*	HT1819	
6.	*Ppdp-1::gfp*	HT1856	Contains the *unc-119* rescue gene
7.	*age-1(hx546)*	TJ1052	
8.	*pdp-1::gfp*	HT1857	Contains the *unc-119* rescue gene Backcrossed to N2 4x
9.	*daf-2(e1370); pdp-1::gfp*	HT1581	
10.	*daf-2(e1370); daf-16::gfp*	HT1531	Padmanabhan et al, 2009 Overexpression of the DAF-16a isoform
11.	*daf-2(1370); Psod-3::gfp*	HT1643	Padmanabhan et al, 2009
12.	*daf-16(mgDf50); daf-2(e1370)*	HT1858	Kwon, et al, 2010
13.	*pdk-1(sa680)*	JT9609	
14.	*daf-2(e1370); akt-1(ok595)*	HT1547	Padmanabhan et al, 2009
15.	*daf-2(e1370); akt-2(ok393)*	HT1548	Padmanabhan et al, 2009
16.	*dpy-5(e907); sIs11033*	BC12915	Transcriptional fusion of the gene F23B12.5 (E2 subunit of PDH) McKay et. al, 2004
17.	*dpy-5(e907); sIs13981*	BC14524	Transcriptional fusion of the gene C04C3.3 (E1β subunit of PDH) McKay et. al, 2004
18.	*daf-7(e1372)*	CB1372	
19.	*daf-14(m77)*	DR77	Inoue et al., 2000
20.	*daf-8(m85)*	DR1631	
21.	*daf-2(e1370); daf-3(mgDf90)*	HT1607	Padmanabhan et al, 2009

Acknowledgements

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Addendum to Chapter 3
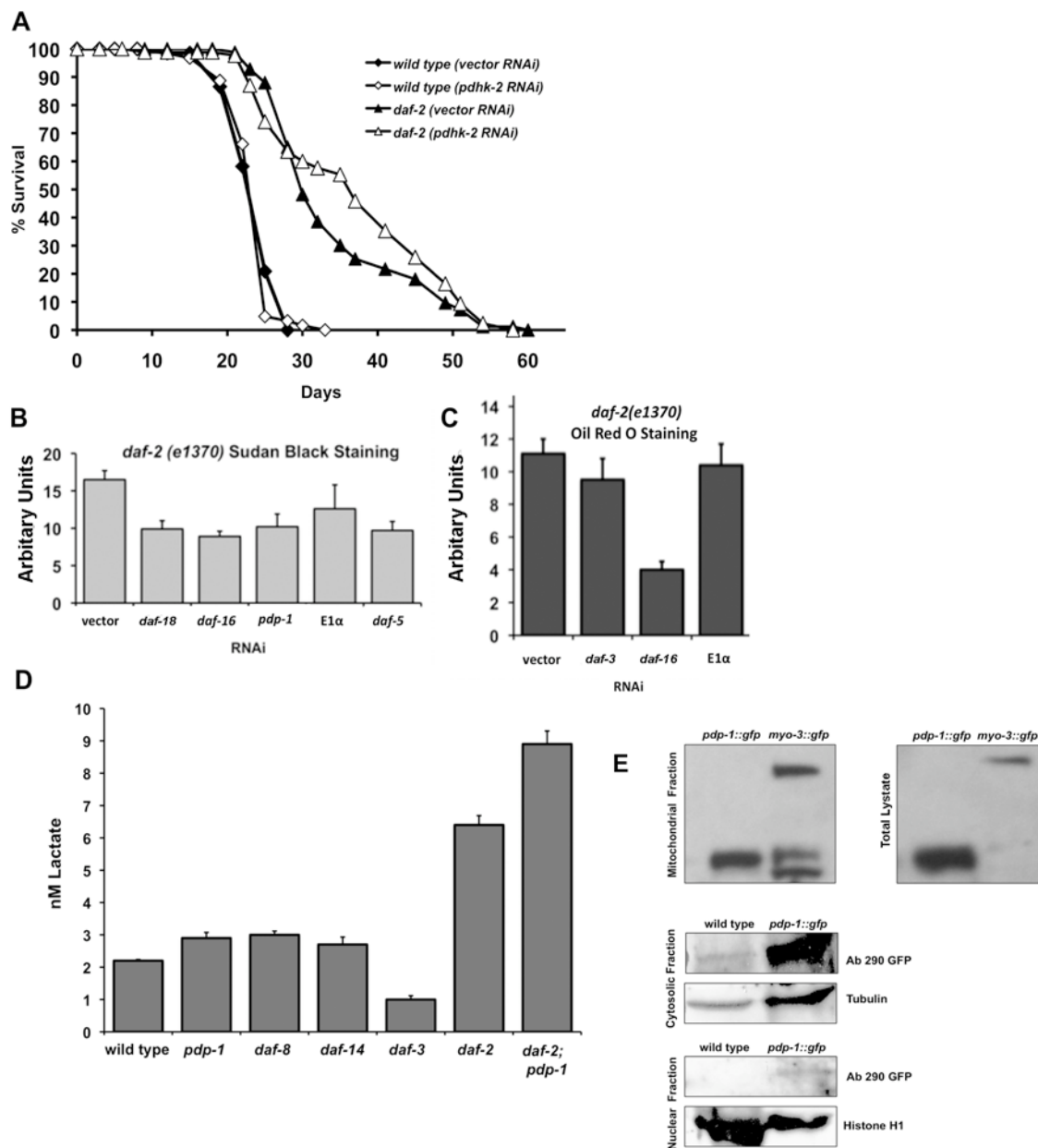
Addendum: Components of the PDHc do not significantly affect phenotypes of the IIS pathway. Data shown is representative of one experiment. All experiments were carried out at least twice.

A) *pdhk-2* RNAi does not significantly reduce the mean and maximal lifespan of wild type worms. A slight extension in the mean lifespan was observed in *daf-2* worms, but this was not seen in an additional repeat. The maximal lifespan of *daf-2* was unchanged on *pdhk-2* RNAi.

B) Quantification of Sudan Black Staining of *daf-2* worms on different RNAi food (n=10). Fat levels were reduced on *daf-18* ($p < 0.001$)*, daf-16* ($p < 0.001$)*, pdp-1* ($p < 0.005$) and *daf-5 (*$p < 0.001$) RNAi respectively.

C) Quantification of Oil Red O Staining of *daf-2* worms on different RNAi food (n=10). Fat levels were significantly reduced on *daf-16* RNAi ($p < 0.001$).

D) Lactate measurements in different mutants of the insulin/IGF-1 and TGF-ß signaling pathways.

E) PDP-1 is broadly expressed in the cell. Fractionation experiments using wild type worms, *pdp-1::gfp* worms and *myo-3::gfp* worms. Upper panel: mitochondrial and cytosolic fractions of *pdp-1::gfp* (75kDa) and *myo-::gfp* worms (110 kDa). As previously reported [38], this strain contains the *myo-3* promoter driving the expression of *gfp* in the mitochondria of muscle cells and was therefore used as a control for the mitochondrial fraction. Middle and lower panel: Cytosolic and nuclear fractions of wild type and *pdp-1::gfp* worms probed with GFP and the appropriate control antibodies.

Preface to Chapter 4

This chapter is a follow-up study based upon the results obtained in Chapter 3. We explore the crosstalk between the insulin/IGF-1 pathway and the TGF-β signaling pathway in greater detail using single and double mutants. Dr. Kelvin Yen, a postdoctoral fellow in the lab performed the lifespan assays in Table 4.1. Ankita Bansal, a graduate student in the lab did the Oil Red O Fat Staining experiments in Figure 4.3. I performed all the other assays in this chapter. This chapter is part of the following manuscript that Dr. Heidi Tissenbaum and I wrote that has been submitted for publication:

Narasimhan SD, Yen K, Bansal A, Padmanabhan S and Tissenbaum HA (2010). PDP-1 Regulates Lifespan, Fat and Development through the Insulin/IGF-1 and TGF-β signaling pathway (Submitted).

Chapter 4:

Investigating the crosstalk between the insulin/IGF-1 and TGF-β signaling pathways in *C. elegans*

Summary

Genetic epistasis studies from the last two decades have identified important roles for the *C.elegans* insulin/IGF-1 signaling (IIS) and TGF-β signaling pathways in modulating dauer diapause. Using single and double mutants of both pathways, it was determined that the IIS and TGF-β pathways acted in a parallel manner to regulate this phenotype. Recently, our lab identified the phosphatase PDP-1 as a novel negative regulator of the IIS pathway. Intriguingly, epistasis studies placed PDP-1 in the TGF-β signaling pathway, suggesting that there was active crosstalk between both pathways. Here we investigate the interaction between both pathways using functional assays. Our studies reveal that TGF-β signaling can intersect with the IIS pathway at multiple levels, and the FOXO transcription factor DAF-16 is likely to be the most downstream mediator of both the pathways. Since the deregulation of TGF-β and insulin/IGF-1 signaling has been implicated in cancer, our studies may provide a new insight into the modulation of these pathways.

Introduction

In *C. elegans,* the decision enter a state of diapause known as dauer is regulated by the processing of sensory cues (food availability, temperature, crowding) that is relayed through the DAF-7 pathway and the DAF-2 pathway that ultimately feed into the DAF-12 nuclear hormone signaling cascade [1]. Molecular cloning of the components of these pathways revealed that the DAF-7 and DAF-2 pathways corresponded to TGF-β-like (BMP) signaling and insulin/IGF-1 signaling (IIS) cascades respectively [2,3]. Both of these pathways show remarkable molecular conservation in higher organisms. The TGF-β superfamily regulates several important biological processes such as cellular growth, differentiation and apopotosis while IIS is a central regulator of longevity, energy metabolism and development [4,5,6].

The identification of these pathways has been primarily through genetic epistasis studies using mutants that either constitutively form dauers *(daf-c)* even in the presence of food or do not form any dauers (*daf-d)* when conditions are unfavorable [1]. The primary conclusions from these studies were that TGF-β signaling and IIS acted in a parallel manner to regulate a common phenotype[3,7]. In the IIS pathway, mutations in the kinases in the pathway, *daf-2, age-1, pdk-1, akt-1* and *akt-2* result in *daf-c* phenotypes, with mutations in the PTEN phosphatase *daf-18* and FOXO transcription factor *daf-16* suppressing this phenotype completely (*daf-d*) [3,8]. Similarly, in the TGF-β signaling pathway,

mutations in the ligand *daf-7,* receptors *daf-1* and *daf-4* and R-Smad proteins *daf-8* and *daf-14* result in *daf-c* phenotypes [2,9]. Dauer formation in this pathway is suppressed by mutations in the co-Smad *daf-3* or Sno/Ski repressor *daf-5* [2,9,10,11].

Recently, we identified and characterized PDP-1, a novel negative regulator of the TGF-β signaling pathway (Chapter 3). Surprisingly, we found that *pdp-1* RNAi/mutation can suppress dauer formation of mutants in the IIS pathway. In addition, it was previously shown that while the IIS pathway regulates longevity, besides dauer formation, the TGF-β signaling pathway had little effect on lifespan [12]. More recent studies have suggested that modulation TGF-β signaling results in slight but significant differences in lifespan [13] Consistent with this, our studies found that as a component of TGF-β signaling was PDP-1 robustly regulated organismal lifespan (Chapter 3). To better understand the crosstalk between IIS and TGF-β signaling, we obtained or generated double mutants of both pathways and performed phenotypic assays on these strains. We specifically focused on the most downstream regulators of both pathways, DAF-3 and DAF-5 in the TGF-β pathway and DAF-18 and DAF-16 in the IIS pathway and asked whether mutations in these genes could modulate signaling through either pathway. We find that the TGF-β pathway can modulate multiple outputs of the IIS pathway, and contrary to the previous conclusions, these pathways are more connected than previously appreciated. Importantly, under conditions of

reduced insulin/IGF-1 signaling, DAF-3 and DAF-5 antagonistically regulate the IIS pathway. Our studies point to distinct roles of DAF-3 in modulating the IIS pathway under normal and reduced signaling conditions. Importantly, modulation of the IIS pathway by components of TGF-β signaling ultimately depend upon DAF-18 and DAF-16.

Results

1. DAF-3 and DAF-5 regulate *daf-2* dauer formation

We first re-investigated the genetic epsitasis analyses of both pathways tested dauer formation as a readout. For the dauer assays described below we used a *ts* allele of *daf-2, daf-2(e1370)*. Normal reproductive growth is observed in this strain at 15°C but at 20°C it forms a significant percentage of dauers [17]. At 25°C, 100% of *daf-2(e1370)* worms form dauers. Enhanced dauer formation by a mutation in the *daf-2* insulin/IGF-1 receptor is suppressed by mutations in the FOXO transcription factor *daf-16* [14,15,16]. Therefore, we wondered what effect would mutations in the Co-Smad *daf-3* and the Sno/Ski repressor *daf-5,* the most downstream components of the TGF-β signaling pathway have on *daf-2* dauer formation (Figure 4.1). Previous studies had used the *e1376* allele of *daf-3* for epistasis analyses, which contains a point mutation [7]. In this study, we also included the *mgDf90* null allele of *daf-3*, which is a deletion of the entire *daf-3* coding region [9]. Since no null alleles are available for *daf-5*, we tested the effect of *daf-5* RNAi besides the *e1386* allele. *daf-2(e1370), daf-2(e1370); daf-3(e1376), daf-2(e1370); daf-3(mgDf90)* and *daf-5(e1386); daf-2(e1370)* mutants were grown at 15°C and dauer formation was assayed at the different temperatures indicated in Figure 4.1. Mutations in both alleles of *daf-3* significantly enhanced *daf-2* dauer formation, with the null allele resulting in almost 100% dauer formation (Figure 4.1A and B). In contrast, a mutation in *daf-5* significantly reduces *daf-2* dauer formation (Figure 4.1C). To further confirm

these results, we grew *daf-2(e1370)* worms on control (empty vector), *daf-3* and *daf-5* RNAi. We observed an even more significant suppression of *daf-2(e1370)* dauer formation using *daf-5* RNAi (Figure 4.1D), suggesting that DAF-5 function is important for dauer formation in these mutants. Importantly, at the restrictive temperature of 25°C, we did not observe a 100% dauer arrest in *daf-5(e1386); daf-2(e1370)* double mutants (Figure 4.1E). Hence under conditions of reduced insulin signaling, such as in a *daf-2* mutant, DAF-3 and DAF-5 have opposite roles in regulating dauer formation. This is in contrast to the function of DAF-3 and DAF-5 in the TGF-β signaling pathway, where they both positively regulate dauer formation.
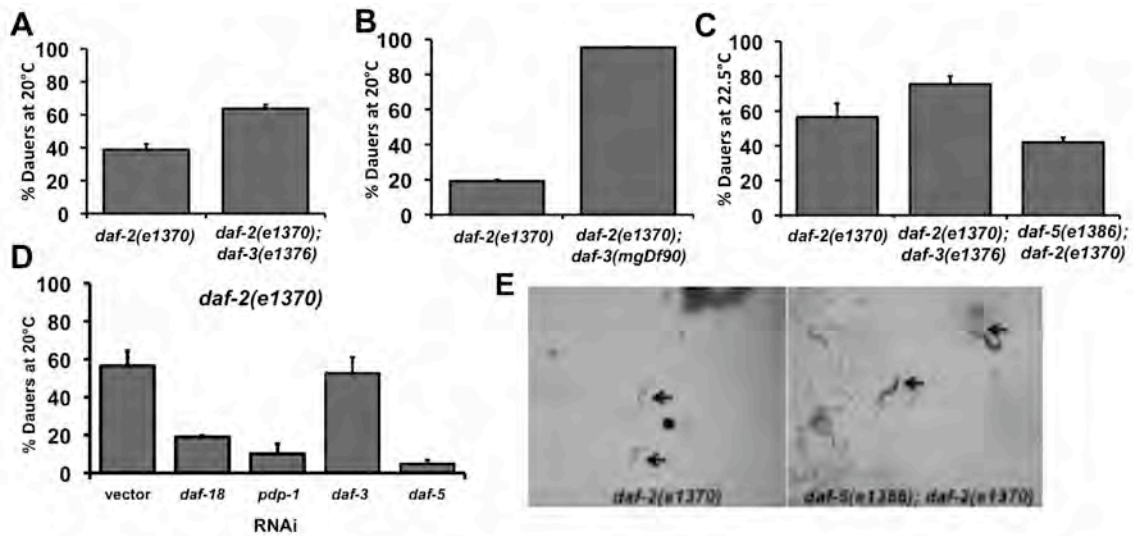
Figure 4.1: DAF-3 and DAF-5 can regulate dauer formation of *daf-2(e1370)* mutants.

Data shown are from one representative experiment. Error bars indicate the standard deviation among the different plates within one experiment.

A) Dauer formation of *daf-2(e1370); daf-3(e1376)* double mutants is significantly enhanced over *daf-2(e1370)* worms (p<0.004).

B) Dauer formation of *daf-2(e1370); daf-3(mgDf90)* double mutants is significantly enhanced over *daf-2(e1370)* worms (p<0.001).

C) *daf-3* (p<0.03) and *daf-5* (p<0.06) mutations enhance and reduce *daf-2* dauer formation.

D) *daf-2(e1370)* dauer formation is reduced on *daf-5* RNAi (p<0.04), similar to *daf-18* and *pdp-1* RNAi.

E) *daf-5; daf-2* double mutants do not arrest as dauers at 25°C while *daf-2* mutants form 100% dauers

2. DAF-16 suppresses dauer formation of TGF-β pathway mutants

We next tested whether mutations in the PTEN phosphatase *daf-18* or FOXO transcription factor *daf-16*, which are negative regulators of the IIS pathway, can affect dauer formation of mutants in the TGF-β pathway. For these experiments, we grew *daf-7(e1372), daf-8(m85)* and *daf-14(m77)* mutants on vector, *daf-18* and *daf-16* RNAi and assayed for dauer formation at the appropriate temperature (Figure 4.2). We find that *daf-16* RNAi can robustly suppress dauer formation of all the three mutants tested (Figure 4.2A, C, E). However, the epistasis results using *daf-18* RNAi were more complex. There was strong dauer suppression of *daf-7(e1372)* and *daf-14(m77)* mutants on *daf-18* RNAi, but there was no effect observed in *daf-8(m85)* mutants (Figure 4.2A, B, D). Taken together, the downstream regulators of both, the IIS and TGF-β pathway can modulate dauer formation of either pathway. This suggests that these two pathways are not parallel or independent. Importantly, since *daf-16* RNAi suppresses dauer formation mutants in the IIS pathway [8] as well as dauer formation of all mutants tested in the TGF-β signaling pathway in this study, it is likely to be the most downstream effector of the two pathways regulating this phenotype.
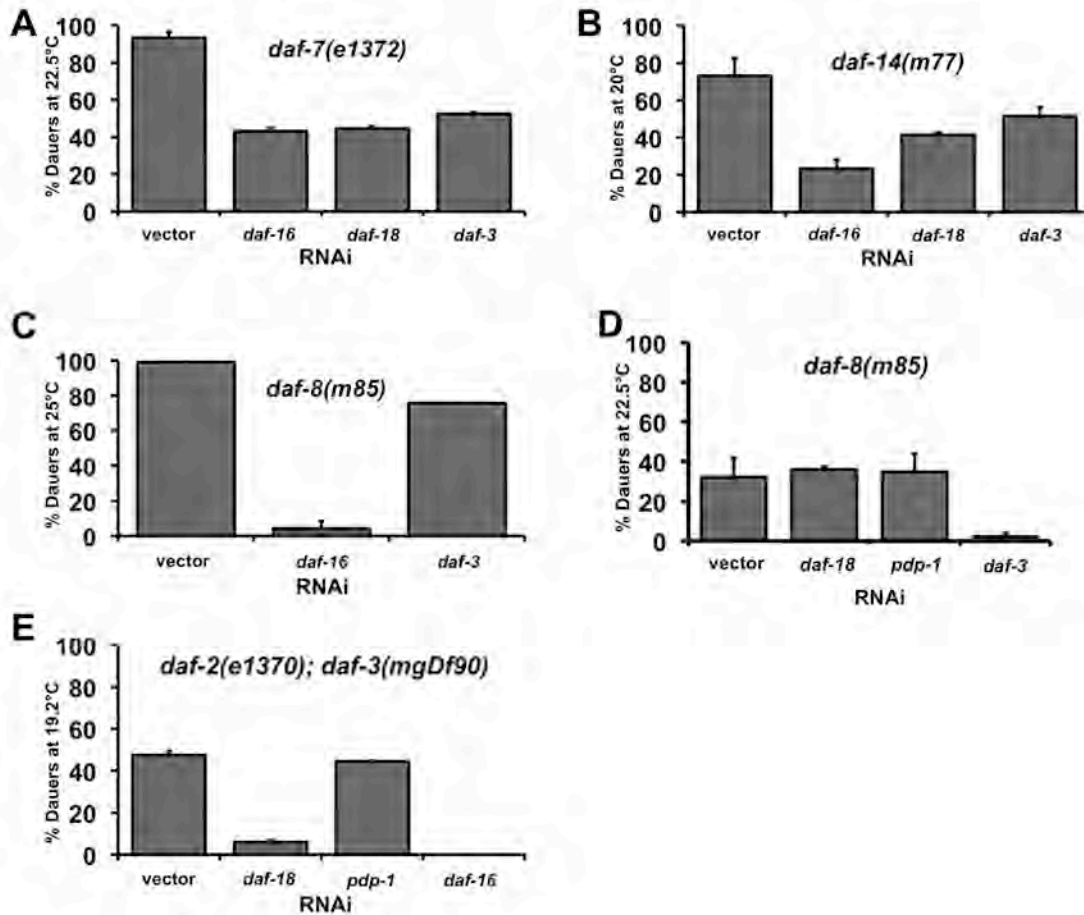
Figure 4.2: DAF-18 and DAF-16 can regulate dauer formation of TGF-β pathway mutants.

Data shown are from one representative experiment. Error bars indicate the standard deviation among the different plates within one experiment.

A) Dauer formation of *daf-7(e1372)* mutants at 22.5°C is significantly suppressed by *daf-18* RNAi (p<0.05) and *daf-16* RNAi (p<0.005), similar to *daf-3* RNAi.

B) Dauer formation of *daf-14(m77)* mutants at 20°C is significantly suppressed by *daf-18* RNAi (p<0.01) and by *daf-16* RNAi (p<0.007), similar to *daf-3* RNAi.

C) *daf-16* RNAi significantly suppresses dauer formation of *daf-8(m85)* mutants at 25°C (p<0.004), but *daf-3* RNAi only has a weak effect.

D) *daf-18* RNAi has no effect on dauer formation of *daf-8(m85)* mutants at 22.5°C, and neither does *pdp-1* RNAi (which has a genetic interaction with *daf-8)*. *daf-3* RNAi can still significantly suppress dauer formation (p<0.005)

E) The enhance dauer formation of *daf-2(e1370); daf-3(mgDf90)* is suppressed by *daf-18* (p<0.04) but not by *pdp-1* RNAi. *daf-16* RNAi results in 100% dauer suppression in this strain.

3. TGF-β signaling regulates longevity through IIS

Besides dauer formation, the IIS pathway also regulates lifespan, stress resistance and fat storage [18,19]. We next tested whether mutations in *daf-3* and *daf-5* could also affect these phenotypes by performing lifespan assays, heat stress assays and fat staining. Single gene mutations in *daf-3, daf-5, daf-18* and *daf-16* result in slight reduction in the lifespan compared to wildtype worms (Table 4.1) [17,20]. In addition, we find that mutations in the upstream components of the TGF-β pathway such as *daf-7* and *daf-14* do not result in lifespan extension (Table 4.1). Similarly, in our earlier studies, found that RNAi or mutation of *pdp-1* results in a very small effect on lifespan. However, *pdp-1* RNAi results in a significant decrease in the lifespan of long-lived mutants of the IIS pathway, such as *daf-2(e1370)* and *age-1(hx546).* Therefore, we next tested whether *daf-3* or *daf-5* mutations or RNAi can modulate the lifespan of these long-lived mutants (Figure 4.3 B and C).   Similar to dauer formation, a null mutation in *daf-3* drastically enhances the lifespan of *daf-2(e1370)* mutants (Figure 4.3B). However, *daf-18* RNAi suppresses lifespan extension in both, *daf-2(e1370)* as well as *daf-2(e1370); daf-3(mgDf90)* mutants, though its suppression of the latter is not as robust. In contrast, *pdp-1* RNAi only has a partial effect on the lifespan of *daf-2(e1370); daf-3(mgDf90)* double mutants, since it acts via the TGF-β pathway. Along similar *daf-5(e1386); daf-2(e1370)* double mutants show a dramatic reduction in lifespan as compared to the *daf-2(e1370)* parental strain, similar to *daf-16 (mgDf50); daf-2(e1370)* double

mutants (Table 4.1). We next tested the effect of a *daf-5* mutation in another long-lived mutant, *age-1*. As shown in Figure 4.3C, *age-1(hx546); daf-5(e1385)* double mutants live significantly shorter than the long lived *age-1(hx546)* parental strain. Again, we find that *daf-18* RNAi can suppress the lifespan of both, *age-1(hx546)* as well as *age-1(hx546); daf-5(e1385)* mutants. Our results suggest that while mutations in the TGF-β pathway by themselves have only small effects on lifespan, components of this pathway are important modulators of longevity under conditions of reduced IIS.

Table 4.1: Lifespans of IIS and TGF-β pathway mutants

Strain	Mean Lifespan at 15°C ± Std. Dev Days	n

Strain	Mean Lifespan at 20°C ± Std. Dev Days	n

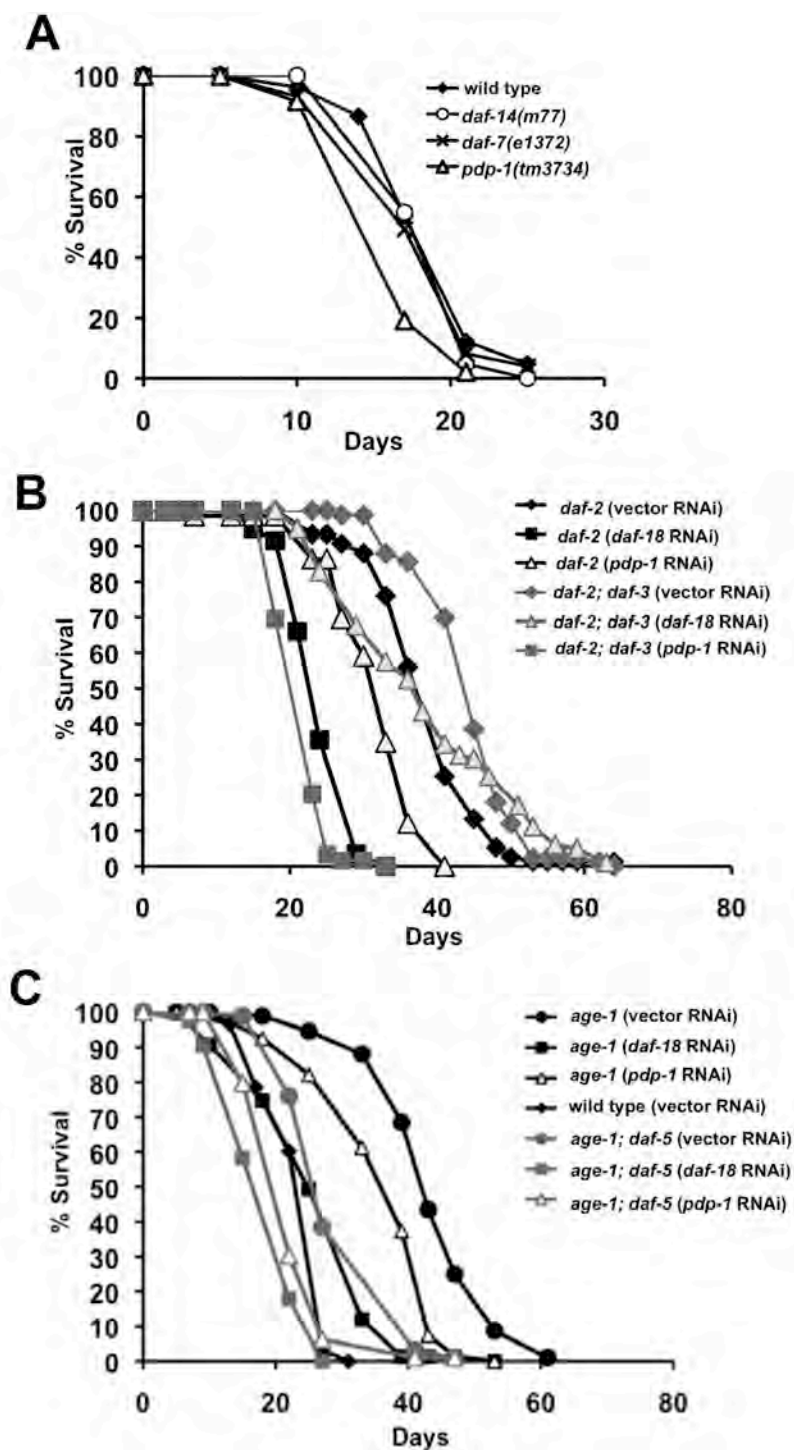| Strain	Mean Lifespan at 25°C ± Std. Dev Days	n

Figure 4.3: DAF-3 and DAF-5 regulate lifespan of long-lived mutants of the IIS

pathway.

A) Mutations in components of the TGF-β pathway do not affect lifespan in *C. elegans*, except for *pdp-1,* which slightly reduces wild type lifespan.

B) Lifespan of *daf-2(e1370); daf-3(e1376)* worms is enhanced over *daf-2(e1370)* mutants (p<0.001). *pdp-1* RNAi can significantly suppress the lifespan of *daf-2(e1370)* worms (p<0.0001) but only has a partial effect on the lifespan of *daf-2(e1370); daf-3(e1376)* worms (p<0.01). *daf-18* RNAi significantly reduces lifespan in both strains (p<0.0001).

C) *age-1(hx546); daf-5(e1385)* double mutants live significantly shorter than *age-1(hx546)* worms (p<0.0001). Both *pdp-1* and *daf-18* RNAi significantly reduce the lifespan of both strains (p<0.0001).

3. TGF-β signaling modulates fat storage and thermotolerance

A number of studies have previously identified roles for the TGF-β pathway in fat storage [16,21]. We next further investigated the crosstalk between both pathways using fat storage as the readout (Figure 4.4). As shown by Oil Red O Staining in Figure 4.4A, mutations in *daf-3, daf-5* and *daf-16* have either slightly less or comparable levels of fat to wild type worms. In contrast, mutations in these genes result in significant changes in the fat storage of "fat" mutants in the pathway such as *daf-2(e1370)* and *age-1(hx546)*. We find that consistent with the lifespan and dauer data, a mutation in *daf-3* slightly enhances *daf-2(e1370)* fat storage while a mutation in *daf-5* results in reduction of fat stores. In fact, *daf-5(e1386); daf-2(e1370)* mutant fat storage was comparable to *daf-16(mgDf50); daf-2(e1370)* worms (Figure 4.4A). Similarly, *age-1(hx546); daf-5(e1385)* mutants stored significantly lesser fat when compared to the parental *age-1(hx546)* strain. We validated the effect of a *daf-5* mutation on *daf-2(e1370)* and *age-1(hx546)* worms fat storage using Sudan Black Staining (Figure 4.4B). Since *daf-16* RNAi could suppress dauer formation of *daf-7* mutants, we tested whether it also affected fat storage (Figure 4.4C). Similar to *daf-3* RNAi, *daf-16* RNAi also results in suppression of fat stores in *daf-7* mutant worms Lastly, we assayed thermotolerance in the single and double mutants of both pathways (Figure 4.4D). The results of the thermotolerance assay were slightly different from the trends observed for the rest of the assays. While a mutation in *daf-5* resulted in

significant reduction in the thermotolerance of *daf-2(e1370)* and *age-1(hx546)* worms, there was no enhancement observed due to the *daf-3* mutation (Figure 4.4D). In addition, we observed that *daf-7(e1372)* mutants showed increased thermolerance compared to wild type worms, almost to the level of *age-1(hx546)* worms.

Taken together, our results suggest that DAF-3 and DAF-5 are important modulators of the IIS pathway. Importantly, DAF-18 and DAF-16 are likely to be the most downstream regulators modulating at least most of the phenotypes of the two pathways, namely dauer fomation, lifespan and fat storage.
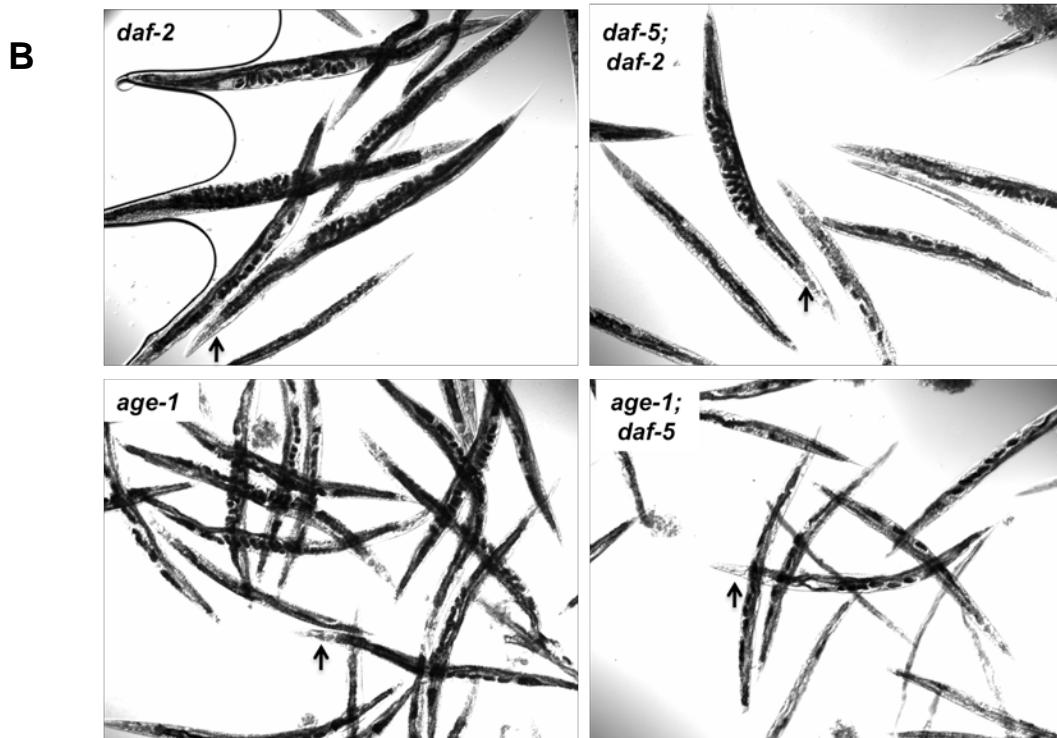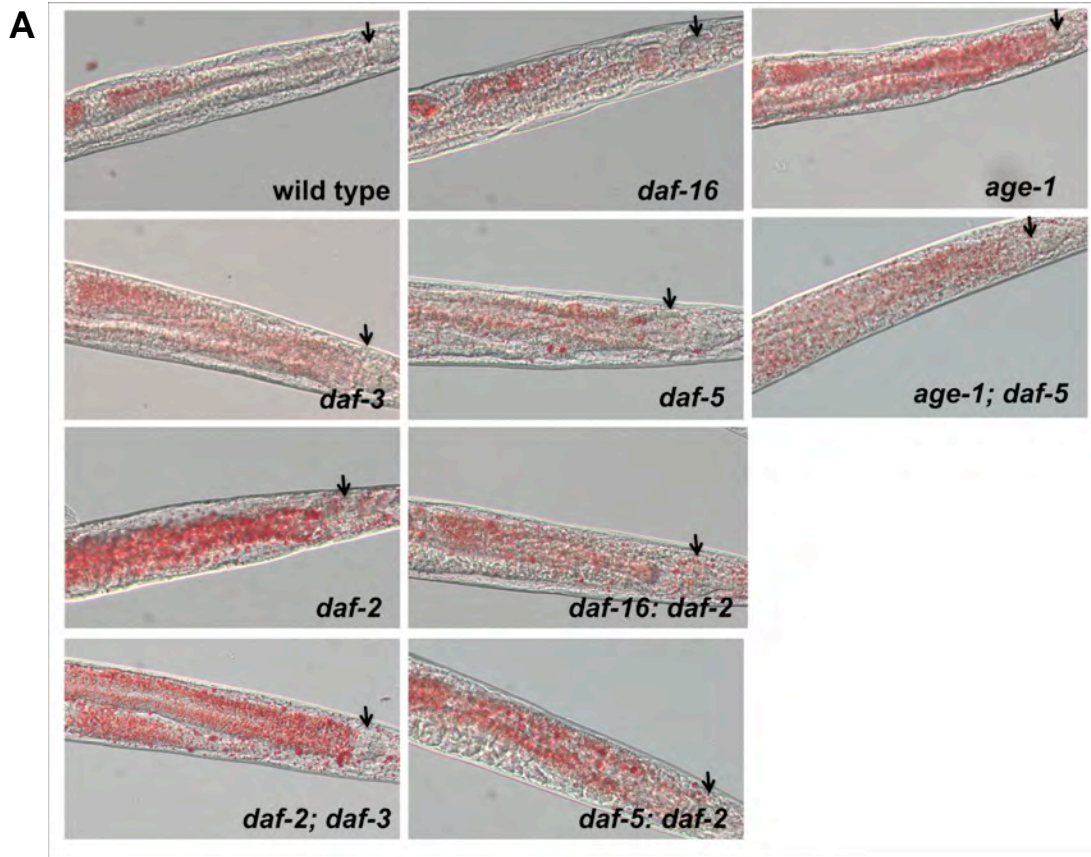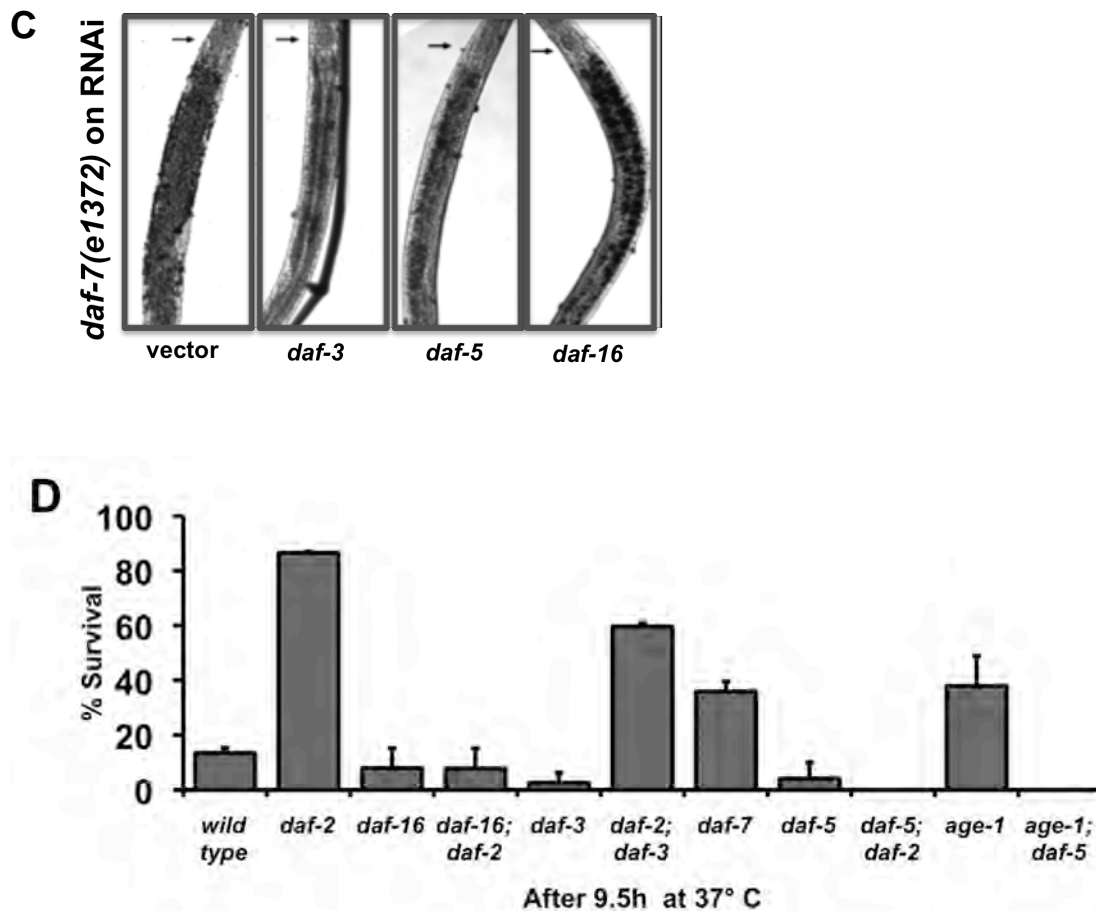
Figure 4.4: Modulation of fat storage and thermotolerance by components of the

TGF-β pathway.

A) Oil Red O Staining in adult worms of the IIS and TGF-β pathways. Arrows

indicate the lower bulb of the pharynx.

B) Sudan Black Staining of adult worms. Arrows indicate the pharynx.

C) Sudan Black Staining of L3 worms of *daf-7(e1372).* Arrows indicate the lower

bulb of the pharynx. Adults were not analyzed as they have an *egl* phenotype,

which results in increased retention of eggs. The eggs can mask the fat staining

phenotype in the intestine.

D) Survival of adult worms of the IIS and TGF-β pathways after 9.5 hours at

37°C. Data shown is an average of two independent repeats.

Discussion

In this study, we identify novel crosstalk between the IIS and TGF-β signaling pathways. Previous studies exploring dauer formation using genetic epistasis analyses had suggested that these two pathways were independent and parallel pathways with little overlap. These conclusions could have been largely due to the unavailability of null alleles and genetic tools such as RNAi. For example, non-null alleles of *daf-16* only partially suppressed dauer formation of TGF-β pathway mutants and therefore DAF-16 was thought to only affect the IIS pathway [7]. Here we re-evaluate the two pathways by looking at the effects of single and double mutants as well as RNAi. First, DAF-3 and DAF-5 were thought to have similar functions and regulation by the TGF-β signaling pathway [22]. In contrast to this, we find that under conditions of reduced IIS, DAF-3 and DAF=5 have opposite functions and they affect various outputs of the IIS pathway in different ways. DAF-3, in particular regulates the IIS pathway in opposite ways, depending upon whether signaling through the pathway is normal or reduced. Importantly, we observed that there is a strong effect of temperature the severity of the phenotypes studied [1]. For instance, the enhancement effect of a *daf-3* null mutation on *daf-2* mutant phenotypes is not seen at 15°C but observed at higher temperatures. Secondly, similar to the previous studies, the TGF-β signaling pathway does not itself have a role in regulating longevity. However, components of this pathway are important regulators of long-lived mutants of the IIS pathway. Consistent with the dauer and lifespan phenotypes,

we find that components of TGF-β signaling can also regulate fat storage under conditions of reduced IIS. Taken together, DAF-3 and DAF-5 are novel modulators of the IIS pathway. Further investigation into how they regulate this pathway will provide a better insight into the crosstalk between both pathways. Previous studies, including our own (Chapter 3) have suggested that the TGF-β signaling pathway regulates the expression of insulins [13,23,24] (Chapter 3). DAF-3 and DAF-5 may either promote or suppress the expression of these insulins, which then feed into the IIS pathway (Figure 4.5).

In addition, IIS and TGF-β signaling may also converge at the transcriptional level, where the SMAD proteins and DAF-16 could co-regulate several of the well-known DAF-16 targets that may act in a combined manner to regulate the phenotypes observed on lifespan, dauer formation, fat metabolism and the response to stress. SMAD proteins have low affinity for binding DNA, and the orchestration of cellular signals into defined outputs requires their association with additional co-factors [25]. Mammalian SMAD proteins can bind several co-activators and co-repressor proteins to modulate gene transcription [4]. Specifically, a synergy between mammalian FOXO (FOXO1, FOXO3a and FOXO4) and SMAD2/3 was identified for the regulation of several genes involved in cell cycle regulation and the response to stress [26]. Importantly, these interactions required the function of the co-SMAD protein SMAD-4, which is homologous to DAF-3 [26]. In a similar manner, DAF-3 and DAF-5 could promote or antagonize DAF-16 directly to regulate the expression of its target genes,

which include antioxidant genes, chaperones and gluconeogenic enzymes [23,27,28]. Despite the different levels of input from the TGF-β signaling pathway, we conclude that DAF-16 is likely to the most downstream effector regulating the various outputs of the IIS pathway.
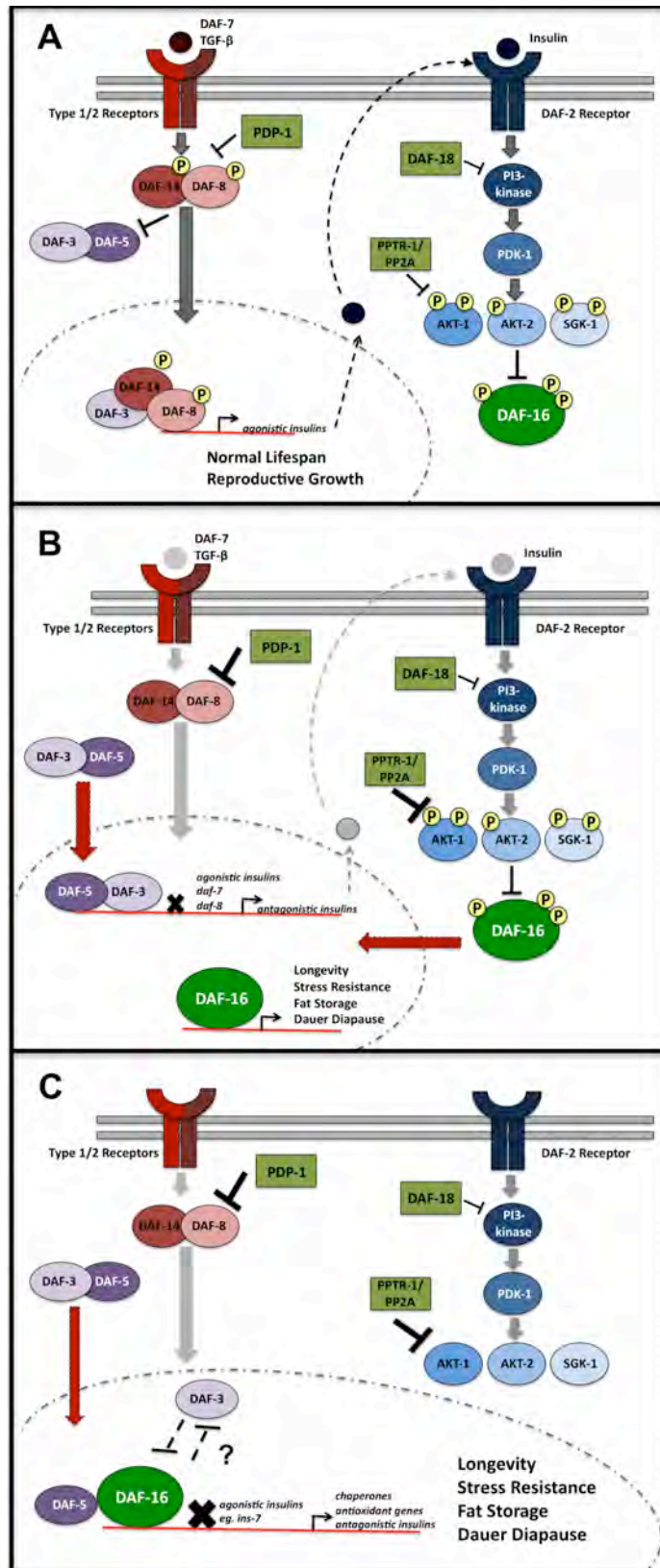
Figure 4.5: Model linking the TGF-β and IIS pathways and highlighting the opposite roles for DAF-3 under different signaling conditions.

(A) Under favorable environmental conditions, signaling through the TGF-ß pathway activates the R-SMAD proteins DAF-8 and DAF-14. The R-SMADs regulate insulin gene expression while antagonizing the activity of the Co-SMAD DAF-3 and the Sno/Ski repressor DAF-5. These insulins may act as agonists and activate the DAF-2 insulin/IGF-1 signaling pathway, thereby promoting phosphorylation and suppression of DAF-16 activity. In this feed-forward model, the worm undergoes reproductive growth and has a normal life span.

(B) The phosphatase PDP-1 negatively regulates TGF-ß signaling through dephosphorylation of DAF-8 and DAF-14. Under these conditions, active DAF-3 and DAF-5 repress the transcription of agonistic insulins. In addition, DAF-3 has also been shown to repress the expression of the *daf-7* TGF-ß ligand and *daf-8*, leading to further downregulation of the TGF-ß pathway. Alternatively, DAF-3 and DAF-5 may also promote the transcription of potential antagonistic insulins. This results in reduced signaling through the IIS pathway, promoting DAF-16 nuclear localization.

C) Under low IIS conditions, DAF-16 localization is predominantly nuclear, where it regulates the transcription of hundreds of target genes that act in combination to regulate longevity, stress resistance, dauer formation and the response to stress. Paradoxically, under low IIS conditions, DAF-3 and DAF-5 play opposite roles. DAF-5 is likely to synergize with DAF-16 and modulate the activity of its

target genes. DAF-3 acts to antagonize DAF-16, either directly or through suppression of DAF-16 target genes. Hence the role of DAF-3 in modulating IIS depends upon the level of signaling through the pathway.

Materials and Methods

Strains

All strains were maintained at 15°C using standard *C. elegans* techniques [29]. Strains used in this study are listed in Table 2.

RNAi based assays

RNAi plates were prepared as previously described [17]. L4 worms were picked onto fresh RNAi plates and maintained for two generations prior to the assay.

Dauer assays

Strains were maintained on RNAi plates for two generations or regular OP50 plates at 15°C. Dauer assays were performed by picking approximately 100 eggs onto 2 fresh plates and incubated at the appropriate temperature. The *daf-7(e1372)* and *daf-14(m77)* worms have a strong Egl phenotype. For dauer assays on these strains eggs were obtained through hypochlorite treatment as previously described [17]. For all dauer assays, plates were scored for the presence of dauers or non-dauers after 3.5-5.5 days, depending upon the strain. Dauer assays were performed at the temperature indicated.

Lifespan Assays

Strains were maintained at 15°C and synchronized by picking eggs onto fresh RNAi or OP50 plates. Approximately 60 young adult worms were transferred per

plate to a total of three fresh RNAi or regular OP50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration of 0.1 mg/ml [30],[31]. All RNAi-based lifespan assays were carried out at 15ºC. Lifespans on OP50 plates were performed at the temperature indicated. Survival was scored by tapping with a platinum wire every 2-3 days. Worms that died from vulval bursting were censored from the analysis. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

Heat Stress assay

Strains were maintained regular OP50 bacteria at 15ºC, as described above. From these plates, approximately 30 young adult worms were picked onto fresh plates and upshifted to 20ºC for 6 hrs. The plates were then transferred to 37ºC and heat stress-induced mortality was determined every few hours till all the animals died.

Fat staining

Strains maintained RNAi on regular OP50 plates were synchronized by picking eggs on to fresh plates and grown synchronously at 15ºC. The plates were then upshifted to 20ºC for 8 hours, at the L2 stage to get L3 worms and at the L4 stage to get young adult worms. The worms were then washed off the plates into microcentrifuge tubes and incubated in 1x PBS buffer for 20 minutes on a shaker at RT. After 2 washes at 3000rpm for 30 seconds with 1x PBS, the strains were fixed according to the type of staining performed. Oil Red O and

Sudan black staining was performed as previously described [32,33,34]. After incubation overnight at RT, worms were mounted on slides and visualized using the Zeiss Axioscope 2+ microscope.

Table 4.2: List of strains used in this study

	Strain	Strain #	Additional information
1.	wild type (N2)		
2.	*daf-2(e1370)*	CB1370	
3.	*age-1(hx546)*	TJ1052	
4.	*daf-16(mgDf50)*	GR1307	
5.	*daf-16(mgDf50); daf-2(e1370)*	HT1858	Kwon, et al, 2010
6.	*daf-7(e1372)*	CB1372	
7.	*daf-14(m77)*	DR77	Inoue et al., 2000
8.	*daf-8(m85)*	DR1631	
9.	*daf-2(e1370); daf-3(mgDf90)*	HT1607	Padmanabhan et al, 2009
10.	*daf-2(e1370); daf-3(e1376)*	HT1608	
11.	*daf-3(e1376)*	CB1376	
12.	*daf-3(mgDf90)*	GR1311	Patterson et al, 1997
13.	*daf-5(e1386); daf-2(e1370)*	JT5486	Vowells and Johnson, 1991
14.	*age-1(hx546); daf-5(e1385)*	JT8045	

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Preface to Chapter 5

In the following chapter, I describe a genetic epistasis study on the phosphatase *fem-2*. This phosphatase was identified in a screen conducted by Dr. Srivatsan Padmanabhan, a former graduate student in the lab.

Chapter 5

The FEM-2 phosphatase regulates insulin/IGF-1

signaling in an allele-specific manner

Summary

Activation of the *C. elegans* insulin/IGF-1 receptor DAF-2 initiates a PI3-kinase signaling cascade that ultimately results in the phosphorylation and negative regulation of the single FOXO transcription factor DAF-16. Negative regulation of the pathway by the PTEN phosphatase homolog DAF-18 or a reduction-of-function mutation in *daf-2* results in enhanced lifespan, fat storage and dauer formation. The components of the IIS pathway have been mostly identified through genetic epistasis studies using dauer formation as a read out. To identify additional negative regulators of IIS, our lab had previously conducted a directed RNAi screen for serine/threonine phosphatases that modulated *daf-2* dauer formation. The top candidate from this screen, *fem-2* robustly suppresses dauer formation when knocked down by RNAi, similar to *daf-18* RNAi. Here we describe additional characterization of *fem-2* and how its modulation of the pathway is specific to the allele of *daf-2* tested.

Introduction

Under favorable growth conditions, *C. elegans* development proceeds normally through four larval stages, L1-L4 to the reproductive adult stage [1]. These conditions include a low pheromone to food ratio as a well as an ambient growth temperature. However, when food is limiting or under higher temperatures, high levels of pheromone can induce developmental arrest in the third larval stage in the form of dauer larvae [1]. The transition to a dauer larva involves several anatomical and physiological changes, such as radial constriction of the body, pharyngeal constriction, modifications in the cuticle and the occlusion of the buccal cavity [1,2]. Dauers store increased fat, are non-feeding, long-lived and resistant to a variety of stresses, including treatment with 1% SDS[1,2]. Early genetic studies in *C. elegans* identified specific pathways that were involved in the regulation of dauer formation [3]. Mutants were identified that would either form dauers despite plentiful food conditions (dauer formation constitutive or *daf-c)* or be unable to form dauers during unfavorable conditions (dauer formation defective or *daf-d)[3]*. Of these, several of the *daf-c* and *daf-d* mutations corresponded to genes that were involved in neuroendocrine pathways such as insulin/IGF-1 signaling (IIS) and TGF-β signaling [1,2]. Interestingly, some of the *daf-c* mutants also showed a significant increase in adult lifespan [4,5].

Genetic epistasis studies identified these long-lived *daf-c* mutants as kinases of insulin/IGF-1 signaling pathway, encoded by the genes *daf-2* (insulin/IGF-1

receptor tyrosine kinase), *age-1* (PI3-kinase), *pdk-1, akt-1* and *akt-2*. Interestingly, two *daf-d* mutations in the genes *daf-18* and *daf-16* could completely suppress the dauer formation and enhanced longevity observed in the kinase mutants, with *daf-18* encoding the lipid phosphatase PTEN and *daf-16* a FOXO transcription factor [6,7,8,9,10,11,12]. Under normal signaling conditions, signaling through DAF-2 results in the negative regulation of DAF-16, thereby regulating growth and reproduction. Negative regulation of the pathway by DAF-18 or under reduced signaling conditions, DAF-16 is more active and translocates into the nucleus to regulate the transcription of genes involved in dauer formation and longevity [13,14,15]. Besides DAF-18, few other negative regulators of the IIS pathway have been identified. Our lab recently performed a directed RNAi screen for serine/threonine phosphatases that would negatively regulate IIS by counterbalancing kinase activity [16] [17]. In this screen, we assayed for the contribution of 60 genes in modulating the dauer formation of *daf-2* mutant. The allele used in this screen, *daf-2(e1370)* is the most commonly used mutant for genetic studies in *C. elegans.* The top candidate from this screen was the gene *fem-2*.

FEM-2 (FEMinization of XX and XO animals 2) has been previously implicated in the regulation of sex-determination in *C. elegans* [18,19]. Wild type worms are hermaphrodites, with two X chromosomes while males, which make up less than 0.1% of the population, have a single X chromosome. Wild type worms undergo spermatogenesis during the L4 stage and the sperm are used to

fertilize the oocytes produced when they become adults[1]. Mutations in *fem-2* affect spermatogenesis and result in "feminization" of both, wild type and male worms[18]. FEM-2 shares approximately 50% homology to mammalian PPM1F, a phosphatase belonging to the PP2C family that has been involved in regulation of calmodulin kinase signaling [20]. In our screen, RNAi of *fem-2* resulted in 100% suppression of *daf-2(e1370)* dauer formation similar to *daf-18* as well as *daf-16* RNAi [16]. Since there were no previous reports linking FEM-2 to dauer formation or the IIS pathway, we looked at the effect of *fem-2* RNAi on a second allele of *daf-2, daf-2(e1368)*. Surprisingly, we observed that *fem-2* RNAi did not have any effect on*, daf-2(e1368)* mutants. Due to this allele-specific phenotype, we proceeded to perform additional characterization of *fem-2* using different alleles of 5 other alleles of *daf-2* besides *e1370.* We confirm that effect of *fem-2* RNAi on dauer formation is only observed in *daf-2(e1370)* and not the other alleles tested. In addition, we describe how the pleiotropy within the different alleles of *daf-2* may explain some of the effects we observe.
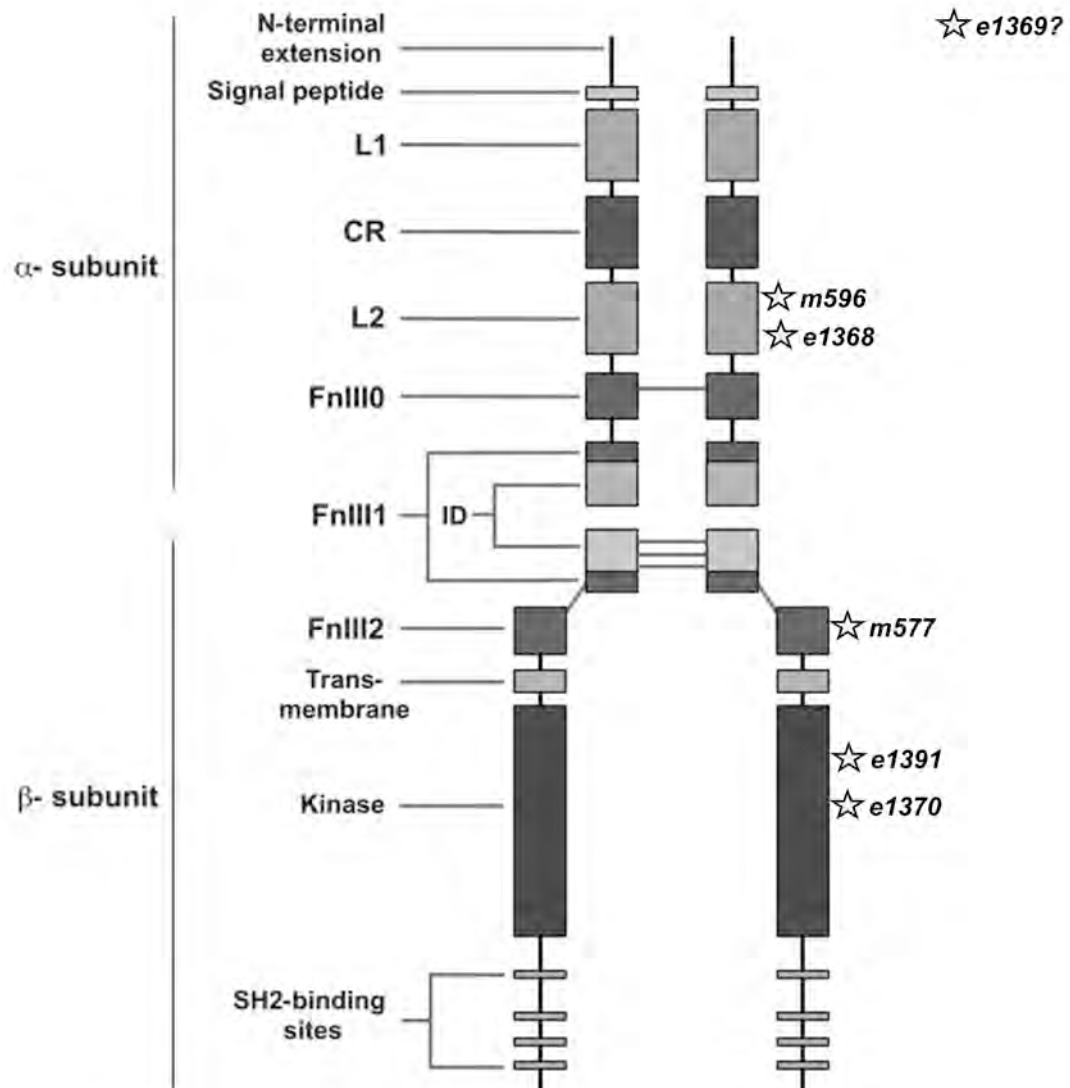
indicate the different mutations that were analyzed in this study. Leucine-rich

domain (L1, L2), Cysteine-rich domain (CR), Fibronectin-type domain (Fn).

(Modified from [5] and [21]).

Results

DAF-2 shares approximately 35% homology with the mammalian insulin and IGF-1 receptors [5]. Similar to mammals, DAF-2 has a N-terminal signal peptide, ligand-binding domain with a cysteine-rich region for interchain disulfide bond formation, a transmembrane domain and a tyrosine kinase domain (Figure 5.1) [5,21]. Several alleles of *daf-2* were identified from early genetic studies, and sequencing analyses have identified that these mutations are found in the extracellular regions and ligand-binding domains as well as the receptor tyrosine kinase domain. [5,21]. The six alleles of *daf-2* used in this study were *daf-2(e1370), daf-2(e1368), daf-2(e1369), daf-2(m577), daf-2(e1391)* and *daf-2(m596)* (Figure 5.1). These mutants can be clustered as following: mutation in the leucine-rich (L2) region *(m596, e1368)*, mutation in the cysteine-rich (CR) region (*m577)* and mutation in the receptor tyrosine kinase domain (*e1391, e1370)*[21]. The mutation in the *e1369* allele does not occur in the coding region [21].

1. *fem-2* RNAi suppresses *daf-2(e1370)* dauer formation

For all assays worms were maintained on the RNAi bacteria for two generations and dauer assays were carried out at the temperature indicated. All assays were repeated at least twice. We first verified the results from our RNAi screen assaying dauer formation of *daf-2(e1370)* mutants on vector RNAi*, daf-18* RNAi and the three top phosphatase candidates, *fem-2* RNAi, *pptr-1* RNAi and *pdp-1*

RNAi (Figure 5.2). The plates were then scored for the presence of dauers and non-dauers animals. As previously reported, dauer formation of *daf-2(e1370)* mutants was robustly suppressed on *fem-2, pptr-1* and *pdp-1* RNAi [16,22]. Of the three phosphatases tested, *fem-2* RNAi had the most severe suppression, similar to *daf-18* RNAi.

A

B

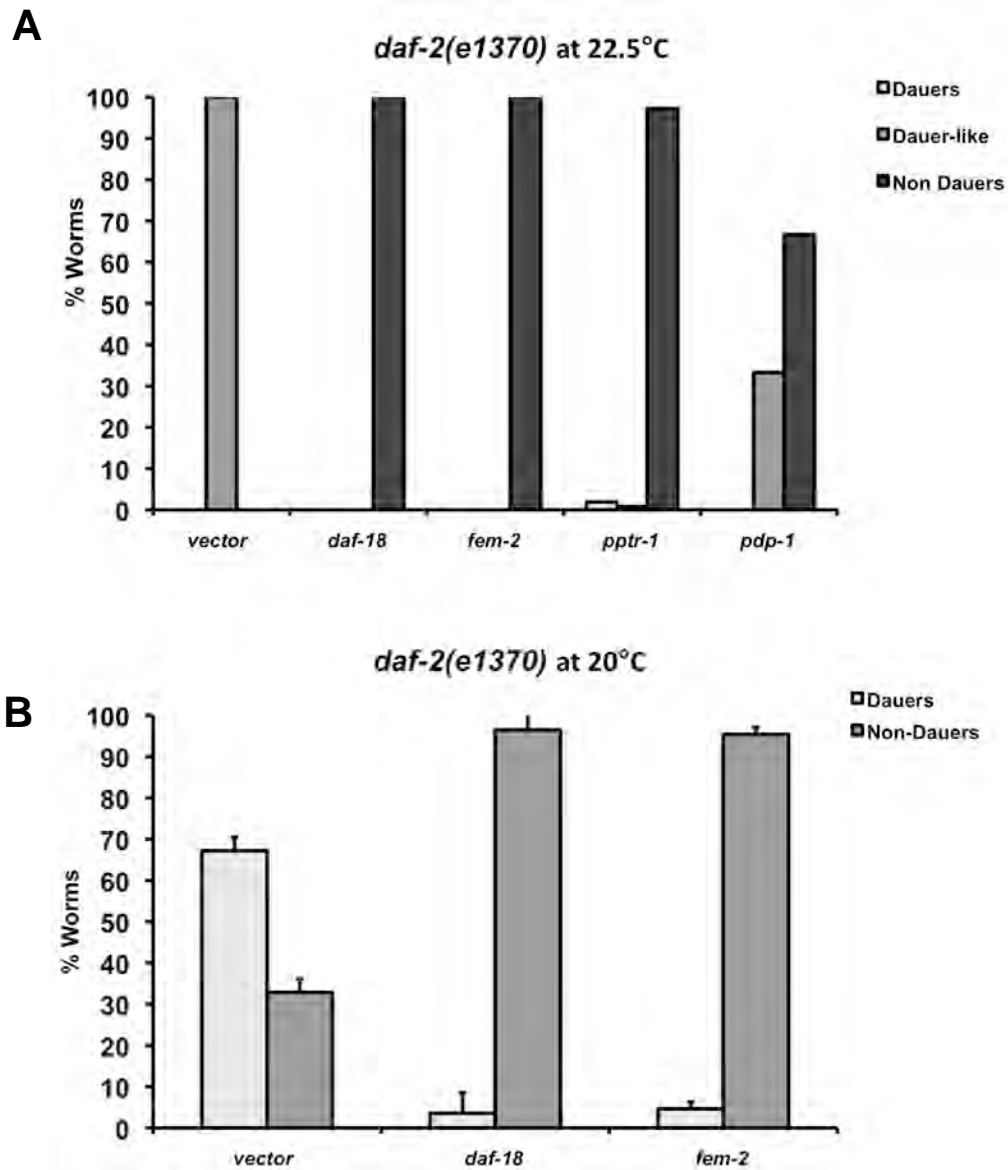

Figure 5.2: *fem-2* RNAi robustly suppresses *daf-2(e1370)* dauer formation

similar to *daf-18* RNAi. Data shown are from one representative experiment. (A)

From our earlier screen, the three top candidates *fem-2, pptr-1* and *pdp-1*

suppressed dauer formation of *daf-2(e1370)* worms. Dauer formation was ~ 88%

on vector RNAi (n=105), ~18% on *daf-18* RNAi (n=102), ~8% on *fem-2* RNAi

(n=142), ~ 34% on *pptr-1* RNAi (n=79) and ~ 42% on *pdp-1* RNAi (n=72). (B) Testing dauer formation of *daf-2(e1370)* at 20°C: dauer formation on vector RNAi was ~ 67% (n=173), on *daf-18* RNAi was ~3.5% (n=242) and on *fem-2* RNAi was ~ 4.6% (n=203).

2. *fem-2* RNAi has a slight effect on *daf-2(e1368)* mutants

To confirm our results, we next tested the effect of *fem-2* RNAi on *daf-2(e1368)* mutants (Figure 5.3). This mutation is considerably weaker than the *e1370* allele of *daf-2*,and dauer formation is observed at a higher temperature of 25°C. To our surprise, *fem-2* RNAi did not have a dramatic effect on dauer formation of *daf-2(e1368)* worms. Quantification of dauers on both sets of RNAi plates revealed that there were fewer dauers on *fem-2* RNAi, however we observed that nearly 20% of the worms in these plates were dauer-like (Figure 5.3). Dauer-like worms have been previously described as larvae with incomplete dauer morphogenesis [23]. These worms share some characteristics with dauers such as growth arrest, reproductive arrest, remodeling of the cuticle but feeding is not completely suppressed and they are not resistant to SDS [23]. We next quantified the different larval stages of the worms on the plates to test whether *fem-2* RNAi had any effect on growth of the worms. On vector RNAi, ~ 30% of the worms were L3s and ~19% were adults, while on *fem-2* RNAi, ~18% were L3s, 14% were L4s and ~31% were adults. These results suggest that *fem- 2* RNAi slightly suppresses the dauer phenotype and increases the growth rate of *daf-2(e1368)* worms. In contrast, 100% of the worms on *daf-18* RNAi were adults. The *e1368* and *e1370* alleles contain mutations in the ligand-binding and the receptor tyrosine kinase domains respectively.

A

daf-2(e1368) at 25°C

B
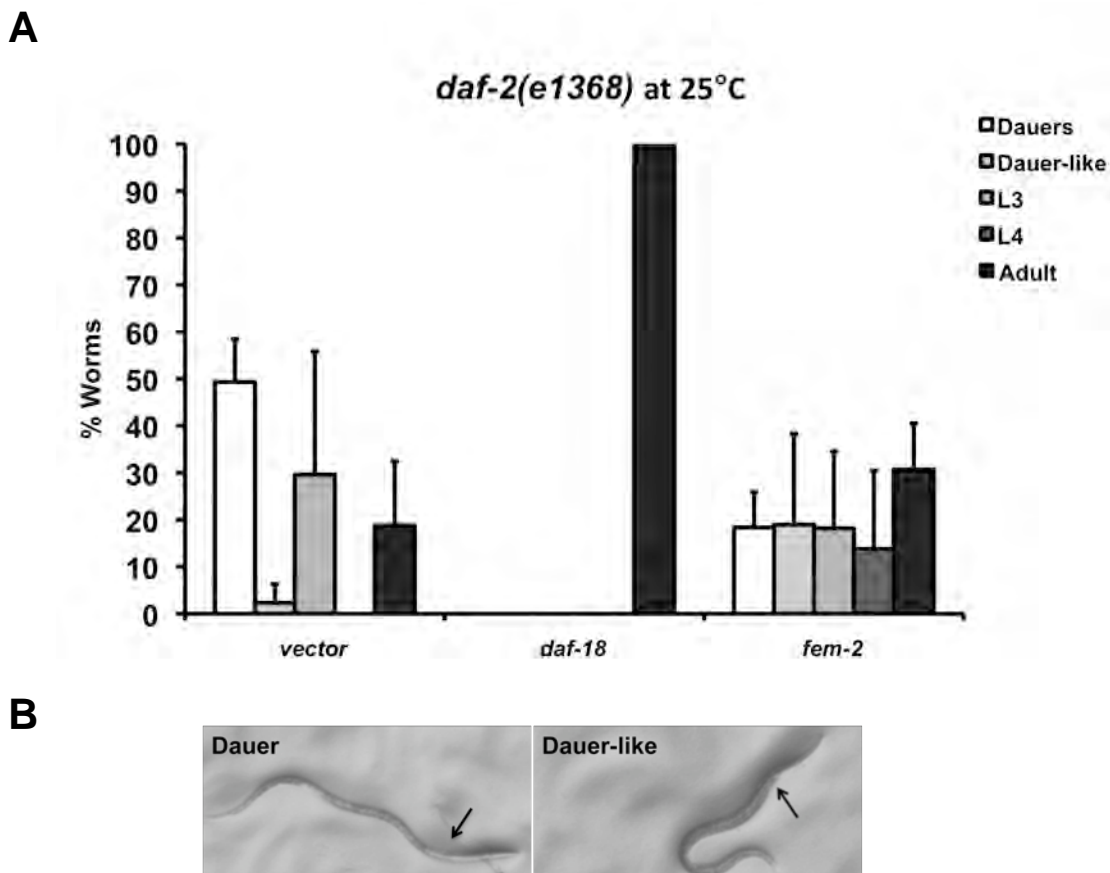
Figure 5.3: A. *fem-2* RNAi slightly reduces dauer formation of *daf-2(e1368)* mutants. Data shown are from one representative experiment. Dauer formation on vector RNAi was ~ 48% (n=130), on *fem-2* RNAi was ~ 18 % (n=79) and on *daf-18* RNAi was 0% (n=70).   Quantification of dauer-like animals was ~2% on vector RNAi and ~19% on *fem-2* RNAi.

B. A dauer larva (left) and dauer-like animal (right). Both are dark and radially constricted, but dauer-like worms are slightly bigger and occasionally show pharyngeal pumping.

3. No effect of *fem-2* RNAi on additional alleles of *daf-2*

We next tested two other alleles, *daf-2(m596)* and *daf-2(e1391),* which contain mutations in the same two domains (Figure 5.1). However we could not find a suitable temperature to assay for dauer formation in both of these mutants. In the case of the *m596* allele, there were no dauers observed any of the RNAi plates at 22.5°C, while at 25°C, all the worms formed 100% dauers. In contrast, the *e1391* allele has an extremely strong *daf-c* phenotype and worms on all the RNAi plates formed 100% dauers at 20°C. At the permissive temperature at 15°C, we could not detectany significant differences in growth and dauer formation was less than 5%. Therefore, we next looked at the effect on *fem-2* RNAi on *daf-2(e1369)* mutants (Figure 5.4). This particular mutation is not found in the coding region of *daf-2*, but instead is thought to lie in a cis-regulatory region [21]. *daf-2(e1369)* mutants show enhanced dauer-formation and an increased mean and maximal lifespan as compared to *e1368* and *e1370* alleles [24]. We observed no difference in dauer formation of *daf-2(e1369)* mutants between vector RNAi and *fem-2* RNAi. In contrast, *daf-18* RNAi resulted in 100% dauer suppression.

The *m577* allele of *daf-2* contains a mutation in the cysteine rich domain of the receptor. Disruption of the equivalent residue in the human insulin and IGF-1 receptors impairs their dimerization as well as localization to the cell membrane [21,25]. We did not observe any significant differences in dauer formation between vector RNAi and *fem-2* RNAi in this allele (Figure 5.5). Quantification of

the larval stages was as follows: on vector RNAi, ~ 1% of the worms were L1/L2s and ~98% were L4s, while on *daf-18* RNAi, ~7% were L1/L2s, ~26 % were L3s and ~67% were adults. On *fem-2* RNAi, ~1% were L1/L2s, 83% were L3s and ~15% were adults, suggesting a delay in growth compared to control. Taken together, *fem-2* RNAi suppresses dauer formation only of the *e1370* allele of the *daf-2* insulin/IGF-1 receptor and has no effect on alleles that bear mutations in different domains of the receptor.

Figure 5.4: *fem-2* RNAi does not affect dauer formation of *daf-2(e1369)* mutants.

Data shown are from one representative experiment. Dauer formation on vector

RNAi was ~ 74% (n=110) and *fem-2* RNAi was ~ 69% (n=76). 100% of worms on

daf-18 RNAi were non dauers (n=142). Since this strain forms a high percentage

of dauers, a significant number were found in the sides of the plate and the lid.

These were not included in the count as it was difficult to assess whether they

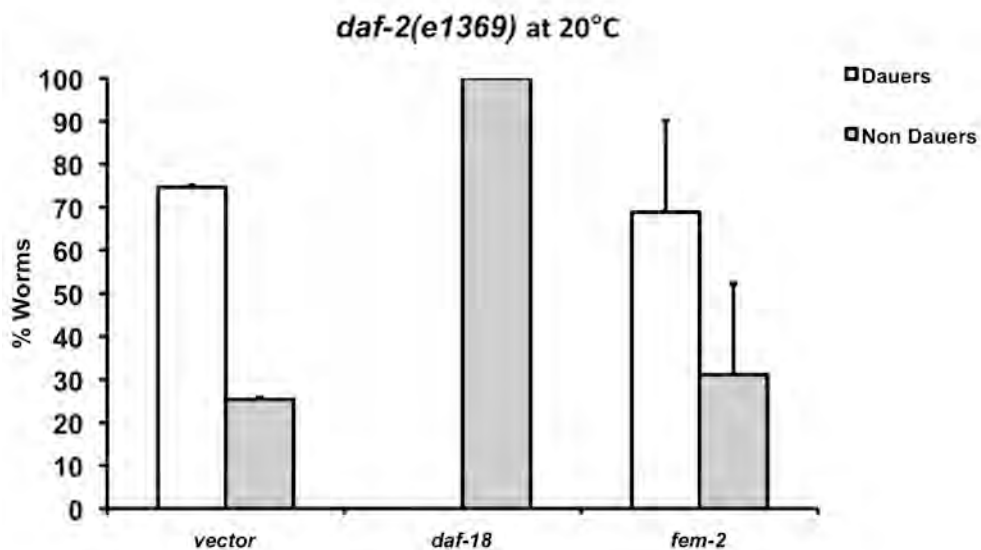had been exposed to the RNAi bacteria.
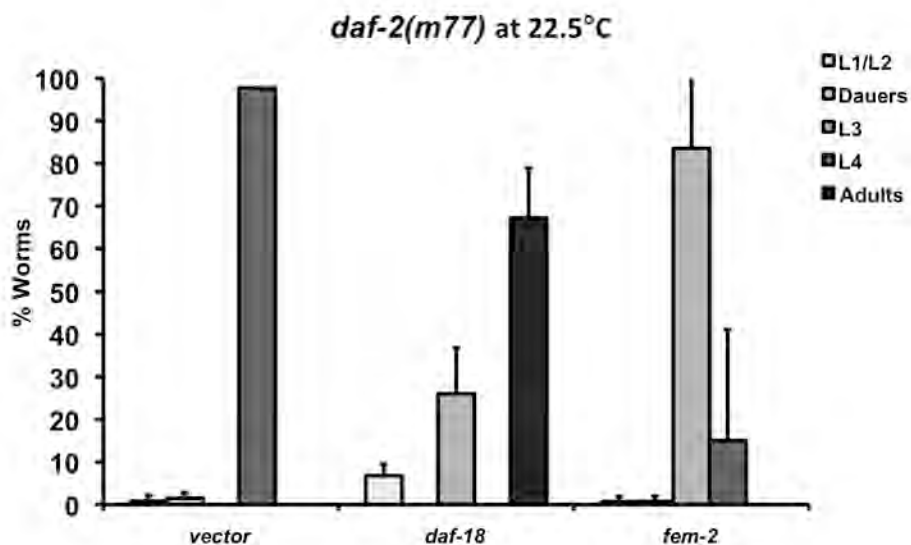
Figure 5.5: *fem-2* RNAi does not affect dauer formation of *daf-2(m577)* mutants.

Data shown are from one representative experiment. Dauer formation of worms

on vector RNAi was ~ 1.5% (n=130), on *fem-2* RNAi was ~1% (n=187) and on

daf-18 RNAi was 0% (n=97). Quantification of the different larval stages shows a

slight delay in growth on *fem-2* RNAi when compared to vector RNAi, with a

majority of the worms on *daf-18* RNAi being non-dauer adults.

Discussion

In this study, we have performed genetic characterization of the serine/threonine phosphatase *fem-2* and its role in regulating the *C. elegans* insulin/IGF-1 signaling (IIS) pathway. FEM-2 was one of three phosphatases identified in a directed RNAi screen for phosphatases that negatively modulated insulin/IGF-1 signaling. RNAi of *fem-2* robustly suppresses dauer formation of *daf-2(e1370)* mutants similar to *daf-18* RNAi [16]. To test whether FEM-2 was indeed a novel negative regulator of (IIS), we performed genetic epistasis analyses using dauer formation as a readout. Multiple alleles of *daf-2* have been identified and the mutations have been mapped to distinct domains of the receptor. Based upon phenotypic differences, the various alleles of *daf-2* have been classified into two classes [24]. Mutants in Class I are *daf-c*, long-lived, thermotolerant and show low levels of L1 arrest at higher temperatures. Class II mutants share these phenotypes and additionally show motility and anatomical defects. Of the mutants tested in this study, *e1368, m596, m577* and *e1369* are Class I alleles while *e1370* and *e1391* are Class II alleles [24]. Interestingly, we observed that *fem-2* RNAi could only suppress dauer formation of the *e1370* allele and had no effect on all of the other alleles tested. The other two candidate phosphatases from the screen, PPTR-1 and PDP-1 did not display any such allele-specific effects (Chapter 2 and Chapter 3). Besides *fem-2*, our lab has observed *e1370* allele-specific dauer suppression with *pyruvate dehydrogenase kinase (pdhk-2)* RNAi (Chapter 3).

This confounding result can be attributed to the pleiotropy associated with the various alleles of *daf-2* itself. The classification of the different alleles were based upon the phenotypic differences mentioned earlier as well as their genetic interaction with the gene *daf-12*. The nuclear hormone receptor DAF-12, homologous to mammalian vitamin D and LXR receptors, is the most downstream regulator of reproductive growth versus dauer diapause [26,27]. Under favorable environmental conditions, neuroendocrine signals from the IIS pathway and other sensory pathways modulate expression of the ligand for DAF-12 through a cytochrome P450 pathway, which upon binding to its receptor, promotes reproductive growth[27]. When conditions are unfavorable, less ligand is produced, and DAF-12 associates with a co-repressor DIN-1 to promote dauer diapause[28]. While a mutation in *daf-16* can suppress the longevity and *daf-c* phenotypes of the different alleles of *daf-2,* a mutation in *daf-12* has allele-specific effects. A *daf-12* mutation suppresses the longevity and *daf-c* phenotypes of Class I alleles while enhancing the same for Class II alleles [21,24]. Similarly, allele-specific effects have been observed for the ribonucleoprotein mutant *rop-1,* which enhances dauer formation of the *e1370* allele but suppresses dauer formation of *m596* [29].

The *m577* and *e1370* alleles, Class I and Class II respectively, are *daf-c* and show an increased lifespan [24]. Under low insulin signaling conditions, such as in a *daf-2* mutant, DAF-16 less phosphorylated and it translocates into the nucleus to regulate the transcription of genes involved in longevity, dauer

formation and metabolism [13]. Interestingly, while this nuclear localization is observed in *e1370* mutants, DAF-16 remains unchanged in *m77* mutants [21]. This difference is likely to be a class difference, as mutations in class I are weaker. In addition, microarray studies have identified more gene changes in *e1370* as compared to the *m596, m577* or *e1368* alleles, and also greater increases in lifespan as well as resistance to hypoxia [30]. These results would support use of *e1370* as the major allele for looking at the effects of reduced insulin/IGF-1 receptor function in worms.

It will be interesting to explore changes that occur downstream of DAF-2, such PI3-kinase activation, AKT phosphorylation and DAF-16 nuclear localization pathway upon *fem-2* RNAi. The effect of *fem-2* RNAi on lifespan of the different alleles will reveal whether the complex epistasis holds true for just one phenotype (dauer formation) or for multiple outputs of the pathway. Lastly, since IIS consists of several branching pathways, it will be interesting to see what happens to other connected pathways such as the Ras pathway or the TOR pathway in the different *daf-2* alleles. Preliminary data in the lab has shown that in the *e1370* allele, phosphorylation of the threonine 350 (308 in mammals) residue of Akt, which is regulated by PI3-kinase signaling, is completely abolished while phosphorylation of serine 517 (473 in mammals), which is regulated by TOR Complex 2, is only slightly reduced (Dr. Kelvin Yen, personal communication). In another example, a mutation in the tyrosine kinase domain in the human insulin receptor, R1174Q results in impaired Ras and MAP-kinase

signaling but normal IRS phosphorylation and PI3-kinase activation [21,31]. If FEM-2 is involved in modulation of IRS signaling or some other yet unidentified adaptor protein that associates with the receptor, it is likely that the *e1370* mutation still allows it access to its substrate compared to the other mutations. Taken together, FEM-2 is a novel modulator of the *C. elegans* IIS pathway and it will be useful to further understand the function of this phosphatase despite its allele-specific regulation, especially given that the *e1370* allele physiologically mimics conditions of reduced IIS.

Materials and Methods

Strains

All strains were maintained at 15°C using standard *C. elegans* techniques [32]. For all RNAi assays, worms were maintained on the RNAi bacteria for two generations before the assays were carried out.

Preparation of RNAi plates

RNAi plates were prepared by supplementing Nematode Growth Media (NGM) media with 100 µg/ml ampicillin and 1 mM IPTG. After pouring, the plates were kept at room temperature (RT) for 5 days to dry. RNAi bacteria were grown overnight at 37°C in LB media supplemented with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline. The next day, the cultures were diluted (1:50) in LB containing 100 µg/ml ampicillin and grown at 37°C until an OD600 of 0.9. The bacterial pellets were resuspended in 1X PBS (phosphate-buffered saline) containing 1mM IPTG. About 200 µl of the bacterial suspension was seeded onto the RNAi plates. The seeded plates were dried at RT for 3 days and stored at 4°C.

Dauer assays

Strains were maintained on RNAi plates for two generations at 15°C. Dauer assays were performed by picking approximately 100 eggs onto 3 fresh plates and incubated at the appropriate temperature. For all dauer assays, plates were scored for the presence of dauers or non-dauers after 3.5-5.5 days, depending upon the strain.

Acknowledgements

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Chapter 6

Discussion and Conclusion

Discussion

The identification of conserved molecular pathways that modulate longevity across several species has undoubtedly provided us with a better understanding of the aging process. In particular, pathways that regulate energy metabolism have been found to play a fundamental role in modulating longevity [1]. These pathways are part of a crisscrossing network containing multiple inputs that ultimately converge upon the insulin/IGF-1 signaling (IIS) pathway [1]. In *C. elegans*, IIS consists of multiple kinases including the DAF-2 insulin/IGF-1 receptor tyrosine kinase, AGE-1 PI3-kinase, the serine/threonine kinases PDK-1, AKT-1, AKT-2 and SGK-1, all of which act to negatively regulate the FOXO transcription factor DAF-16 [2]. Under reduced IIS conditions, DAF-16 activates the transcription of hundreds of target genes that regulate longevity, metabolism, stress resistance and dauer diapause [3,4,5,6].

Aging is associated with a decline in the ability to maintain nutrient and signaling homeostasis, both at the cellular and systemic level. Importantly, aberrant signaling through the IIS pathway is implicated in several age-associated diseases including type 2 diabetes, cancer, obesity and neurodegenerative disorders [7,8,9,10,11]. When this study was started, many of the kinases in the pathway and their regulation of DAF-16 had been well studied. However, not much was known about negative regulators of the pathway. In particular, except for the PTEN phosphatase DAF-18, which antagonizes PI3-kinase activity, no phosphatases had been identified for other kinases in the

pathway[2,12,13,14]. In addition, there was no report of a phosphatase for DAF-16, which itself is regulated by multiple kinases within the IIS pathway and other independent pathways [15]. The majority of the phosphorylation events in a cell occur on serine/threonine residues, but only a small pool of serine/thronine phosphatases presumably act to dephosphorylate hundreds of substrates [16]. We were therefore interested in identifying phosphatases that counterbalanced serine/threonine phosphorylations in the IIS pathway. My work has focused on the identification and characterization of three novel phosphatases that negatively regulate IIS through distinct mechanisms. Through these studies, we also identify a role for the TGF-β signaling pathway in modulating IIS.

PPTR-1 and PDP-1: Novel negative regulators of the IIS pathway

In Chapter 2 and 3, we describe the identification and characterization of two novel negative regulators of the IIS pathway. Protein Phosphatase Two A Regulatory Subunit -1 (PPTR-1) and Pyruvate Dehydrogenase Phosphatase homolog -1 (PDP-1) were two top candidates identified from a directed RNAi screen for serine/threonine phosphatases that modulate *daf-2(e1370)* dauer formation. Similar to *daf-18* RNAi, *pptr-1* RNAi and *pdp-1* RNAi results in significant suppression of dauer formation. Besides dauer formation, PPTR-1 and PDP-1 also regulate longevity, stress resistance, fat storage and positively modulate DAF-16 activity. Therefore, PPTR-1 and PDP-1 are major negative regulators of the IIS pathway. It is currently unclear how these proteins are

themselves regulated. We observed that *pptr-1* RNAi and *pdp-1* RNAi had only small effects on the lifespan, stress resistance and fat storage of wild type worms, but saw dramatic effects on these phenotypes in a reduced IIS background, such as in the long-lived hypomorphic mutants *daf-2* and *age-1* [17]. Yet when signaling was entirely abrogated, we find no effect on lifespan. Therefore, it is likely, that a small threshold of signaling is required for the optimal function of PPTR-1 and PDP-1 in modulating the pathway. The level of substrate could be a limiting factor for the activation and requirement of these phosphatases to modulate the IIS pathway. Under severely reduced signaling conditions, DAF-16 is almost entirely nuclear, and therefore loss of PPTR-1 and PDP-1 are likely to not affect outputs of the pathway. Instead, both of these proteins are more critical when signaling through the pathway needs to be fine-tuned and downregulated to promote survival under conditions of nutrient or environmental stress during the dauer stage or as an adult.

Despite these similarities, the mechanisms through which these phosphatases regulate IIS are starkly different. PPTR-1 is part of holoenzyme consisting of a broadly expressed catalytic (PP2Ac) and structural subunit (PP2Aa) [2]. PPTR-1 negatively regulates AKT-1 by modulating its dephosphorylation at a conserved threonine (T350 in worms/ T308 in mammals). Remarkably, this molecular interaction has also been observed in mammalian cells and in an independent study in *Drosophila* [2,18]. In contrast, we observe that PDP-1 acts at the level of the R-SMADs DAF-8 and DAF-14, that are part of

the DAF-7/TGF-β signaling pathway, and therefore PDP-1 indirectly modulates IIS and its multiple outputs. This genetic interaction has also been complemented by biochemical studies in *Drosophila* and mammalian cells, suggesting that in higher organisms PDP-1 acts to dephosphorylate the R- Smad protein SMAD-1 to negatively regulate TGF-β signaling [19]. It is interesting to note how a component of TGF-β signaling can so robustly modulate IIS. Several interesting questions remain to be answered with regards to both phosphatases.

PPTR-1, as a regulatory subunit, provides substrate specificity for the PP2A holoenzyme which otherwise has hundreds of cellular targets [20]. Besides AKT-1, the closely related SGK-1 can also interact with and be dephosphorylated by PPTR-1/PP2A (Dr. Kelvin Yen, personal communication) [17,21]. The tissue expression patterns of PPTR-1 and AKT-1 have little overlap with SGK-1. Additionally, we found no expression of PPTR-1 and AKT-1 in the intestine, which is the major tissue for the regulation of lifespan by DAF-16 [22]. In contrast, SGK-1 is broadly expressed in the intestine [17,23]. Because both AKT-1 and SGK-1 directly phosphorylate DAF-16 [23], it is possible that PPTR-1 regulates the activity of these kinases in a cell or tissue-specific manner. By using longer promoters for the transgenes tested in this study or examining the tissue expression patterns of PPTR-1 under different conditions (reduced IIS, heat stress etc), we may be able to address whether the PPTR-1-SGK-1 interaction indeed occurs *in vivo.*

In mammals, hyperphosphorylation of Akt has been linked to cancer, while the hypophosphorylated form is associated with Type 2 diabetes [24,25]. Maximal activation of Akt occurs through phosphorylation at T308 by PDK-1 and at S473 by TORC2 [26,27]. While PPTR-1/PP2A regulates T308 (350 in worms) dephosphorylation the PHLPP phosphatases dephosphorylate the S473 (517 in worms) residue [17,28]. The PHLPP phosphatase homolog has not been characterized in worms. It will be interesting to see whether dosage modulation of this phosphatase can also regulate IIS in the context of a whole organism, similar to PPTR-1 and whether it involves crosstalk with the mTOR pathway. Our study observed that dephosphorylation of T350 was enough to promote DAF-16 activity and regulate longevity. In contrast dephosphorylation of the serine residue of Akt in mammals had no effect on Serine 256, the site in FOXO that is the critical determinant in its transactivation [29,30]. Therefore, differential regulation of dephosphorylation at distinct residues within a single protein, in this case Akt, can have pleiotropic effects on outputs of the IIS pathway. PPTR-1 was the only one of seven regulatory subunits in worms that affected dauer formation. Testing the other regulatory subunits for their roles in modulating other outputs of the pathway may reveal specific substrates for PP2A.

The identification of PDP-1 in the screen as potential modulator of IIS was interesting and unexpected. In mammals, it was already established that PDP dephosphorylated the E1α subunit of the pyruvate dehydrogenase complex (PDHc), resulting in activation of the enzyme complex [31]. Yet in our study,

RNAi of the components of the pyruvate dehydrogenase complex (PDHc) in worms does not affect dauer formation. This suggested to us that perhaps there might be an independent role for PDP-1 in modulating the IIS pathway. Interestingly, recent studies have identified additional substrates and roles for metabolic enzymes in modulating cellular signaling, with the notable examples being GSK3 and GAPDH [32,33]. Despite PDP-1 regulating multiple aspects of IIS, using genetic epistasis, we identified it to be acting in the DAF-7/TGF-β signaling pathway. Consistent with previous studies establishing a role for TGF-β signaling in regulating insulin gene expression in worms and mammals, we find that the levels of several agonistic insulins are increased in *pdp-1* mutants [34,35,36]. Therefore, in our model PDP-1 downregulates the TGF-β signaling to reduce the expression of insulins, which subsequently prevents activation of the IIS pathway. Generating phospho-specific antibodies in worms will be useful to confirm the dephosphorylation of DAF-8 and DAF-14 by PDP-1. Importantly, because deregulation of TGF-β signaling has been implicated in cancer in humans, it will be interesting to test the levels and activity of PDP in biopsy samples. In addition, it will be explore any correlation between changes in R-SMAD phosphorylation status and Type 2 diabetes.

Mitochondrial dysfunction has been associated with changes in longevity in worms and higher organisms [37]. It has not been possible thus far to entirely uncouple PDP-1's role in regulating PDH activity in the mitochondria from modulation of the TGF-β pathway. Preliminary data using imaging and

fractionation suggests PDP-1 is enriched in the mitochondria but also expressed in the nucleus and cytosol. The protein sequence of PDP-1 contains a strong mitochondrial targeting signal, so it is currently unclear what could account for its distribution in other cellular compartments. Based upon current annotations, there are no additional *pdp*-like genes in the worm, and no additional isoforms of *pdp-1*.

Similar to humans, a mutation in *pdp-1* results in increased levels of lactate in the worm. However, this increase in lactate alone may not account for its role in modulating various phenotypes of the IIS pathway, as mutants of the DAF-2 and DAF-7 pathways also show elevated levels of lactate. Interestingly, dosage modulation of PDP-1 results in drastic changes in fat storage in worms. Therefore, it will also be useful to test whether PDP-1 directly affects pathways that regulate adipogenesis or lipolysis. Unlike PPTR-1, which presumably has no enzymatic activity, PDP-1 itself contains the catalytic domain. A regulatory subunit had been characterized for bovine PDP but a BLAST search did not provide any highly homologous protein in worms [38]. It is therefore possible that besides regulating PDHc and TGF-β signaling, PDP-1 has additional cellular substrates. Further studies by immunoprecipitating PDP-1 and carrying out proteomic analyses such as using multidimensional protein identification technology (MUDPIT) can enable us to verify as well as identify PDP-1 substrates as well as potential regulatory partners that determine substrate specificity or regulate its activity.

Linking TGF-β signaling and IIS

In Chapter 4, we investigate the crosstalk between IIS and TGF-β signaling pathways. Genetic epistasis studies from the last two decades had concluded that in *C. elegans,* IIS and TGF-β signaling were two independent pathways with little overlap [39]. In contrast to these conclusions, we discovered that like PDP-1, other components of the TGF-β signaling pathway, notably the Co-SMAD DAF-3 and Sno/Ski repressor DAF-5 could robustly regulate longevity, dauer formation, stress resistance and fat storage. Interestingly, similar to *pdp-1*, mutations in *daf-3* and *daf-5* do not significantly affect these phenotypes. However, under conditions of reduced IIS, DAF-3 and DAF-5 regulate multiple outputs of the pathway in opposite ways. It will be interesting to explore how the synergy or interaction between DAF-3, DAF-5 and DAF-16 feeds into the DAF-12 nuclear hormone receptor, which is the main downstream effector required for dauer formation. As discussed earlier, a feed-forward model suggests that TGF-β signaling regulates the expression of insulin(s), which can then bind the DAF-2 insulin/IGF-1 receptor and activate the IIS pathway [36]. However, there are 40 insulin-like genes in worms, and only a few such as *ins-1, daf-28* and *ins-7* have been shown to affect phenotypes of the IIS pathway [5,36,40,41,42]. Studies are ongoing in this laboratory to understand the expression and regulation of the insulin genes at a systems level. An alternative model suggests a direct transcriptional response, where SMAD proteins can either activate or antagonize DAF-16. Indeed, a FOXO-SMAD synergy has been previously reported using

mammalian cell culture, where both transcription factors regulate cell cycle and stress-related genes in combination [43]. The use of phospho-antibodies against proteins in both pathways (phospho-SMAD, phospho-Akt and phospho-DAF-16) will help to elucidate where and how these pathways intersect. We are currently interested in carrying out microarray studies and chromatin immunoprecipitation (ChIP) experiments to identify common and distinct targets of DAF-3 as well as DAF-16.

Allele-specific regulation of IIS

We describe the characterization of the phosphatase FEM-2 in Chapter 5. From the original RNAi screen, *fem-2* RNAi resulted in the most severe suppression of *daf-2* dauer formation, comparable to *daf-16* RNAi [17]. Yet this phenotype was only observed in in the *e1370* allele, of the six alleles that were tested. Unlike *pptr-1* and *pdp-1*, which showed no allele-specificity, *fem-2*'s regulation of IIS was allele-specific, which lead us to question whether FEM-2 indeed has a biological role in this pathway. Besides *fem-2,* we also observed a similar phenotype with the gene *pdhk-2*, which is homologous to mammalian pyruvate dehydrogenase kinase (PDHK). In mammals, PDHK phosphorylates the $E1\alpha$ subunit of PDHc and inactivates it [44,45]. PDHK is closely linked to the IIS pathway, with levels of this kinase elevated under reduced signaling conditions [45]. Additionally, PDHK is a direct target of FOXO [46]. We observed that RNAi of *pdhk-2* results in suppression of dauer formation of *daf-2(e1370)* worms.

However, there was no effect of *pdhk-2* RNAi on a second allele of *daf-2* tested, *daf-2(e1368)* or in *pdk-1* mutants.

This apparent paradox can be explained in part by the fact that the different *daf-2* alleles do not behave similarly and provide somewhat opposite phenotypes in some double mutant backgrounds [47,48]. The mutations fall in different domains of the receptor, and while some alleles may allow ligand-binding and partial activation of the pathway, others may result in complete abrogation of signaling. As a consequence, in the different alleles of *daf-2*, distinct downstream components may be activated or inactivated. In addition, a majority of the mutants used in the genetic epistasis analyses in the past have been non-null (including *daf-2, age-1, pdk-1),* which is understandable to some extent as null alleles result in larval lethality. Microarray studies comparing the *e1370* allele, which contains a mutation in the receptor tyrosine kinase domain, to other alleles have identified more changes in the former. In addition, *e1370* is the most widely used allele for understanding the effects of reduced IIS in *C. elegans,* with the results of several studies including our own also showing molecular conservation in higher organisms. It is therefore likely, that the allele-specific phenotypes observed using *fem-2* RNAi and other genes such as *pdhk-2* RNAi are biologically relevant. Importantly, while genetics has been a powerful tool for studying IIS and the biology of aging in *C. elegans*, using biochemical approaches such as assaying for PIP_2/PIP_3 levels, AKT phosphorylation or DAF-

16 activity will most certainly complement and strengthen any conclusions derived from epistasis analyses.

Conclusion

This study unravels three novel phosphatases that negatively modulate IIS and are likely to be important for maintaining signaling homeostasis. Additionally, we demonstrate that the TGF-β signaling pathway can intersect with IIS at multiple levels. Importantly, modulation of IIS by the phosphatases and input from TGF-β signaling ultimately converge on the regulation of DAF-16. These studies provide a new perspective on our understanding of IIS as well as the regulation of longevity and metabolism in the context of a whole organism.
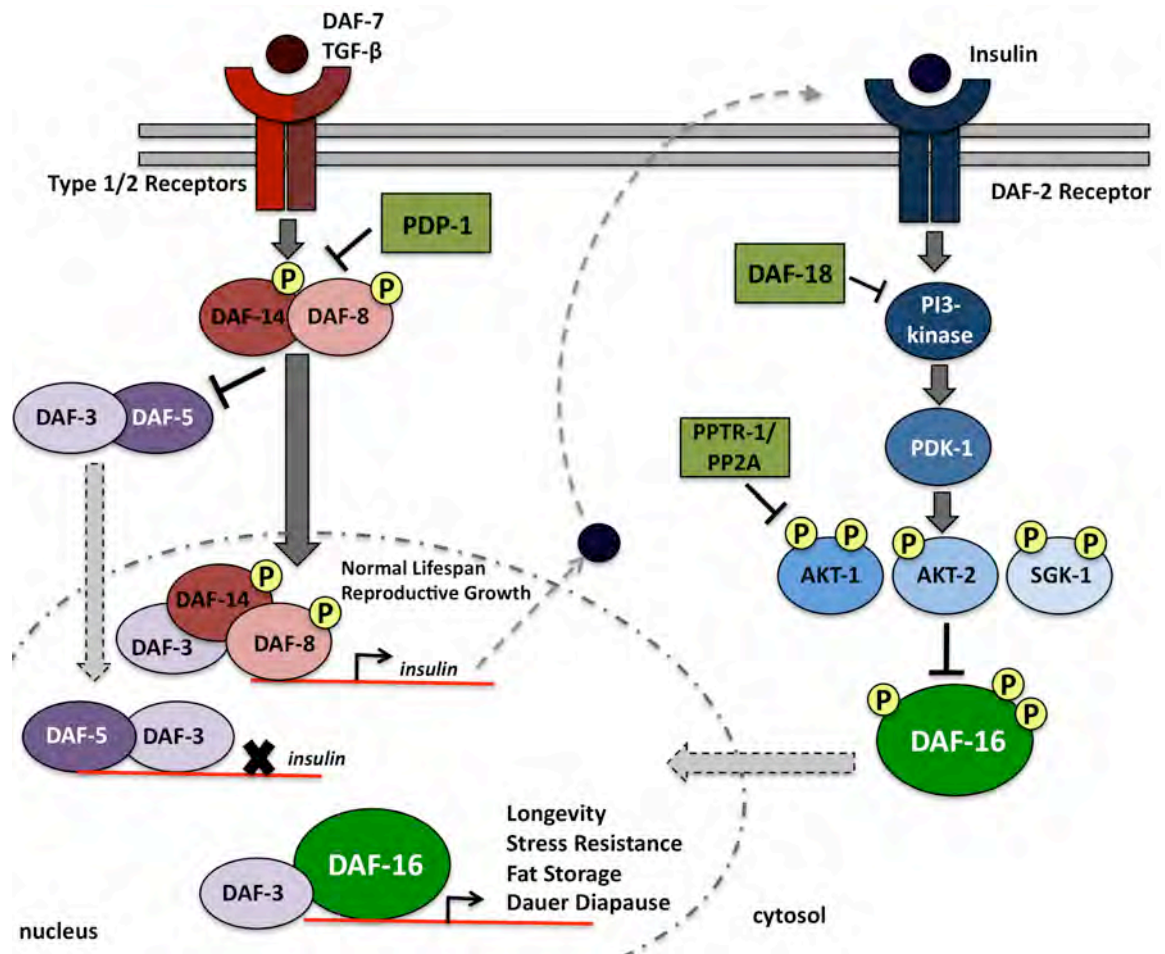
Figure 6.1: Model summarizing the major findings of this study. The serine/threonine phosphatases PPTR-1/PP2A and PDP-1 (green rectangles) negatively regulate the IIS pathway either directly, through AKT-1 dephosphorylation, or indirectly, through downregulation of TGF-β signaling. The latter results in reduced expression of insulin gene expression and therefore reduced activation of the DAF-2 insulin/IGF-1 receptor. Recduced signaling through the IIS pathway promotes DAF-16 activity, resulting in longevity, stress resistance, increased fat storage and dauer diapause. Downregulation of TGF-β

signaling also activates DAF-3 and DAF-5, which can potentially synergize with or antagonize DAF-16 to regulate its various outputs.

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