THE DYNAMIC GUT: NUTRIENT REGULATION OF AGING AND DEVELOPMENT BY PHA-4/FOXA

by

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ABSTRACT

The gastrointestinal (GI) tract is a central player in the regulation of development and aging. FoxA factors are master regulators of the GI tract during embryogenesis and after birth but little is know about their regulation. I use the simple digestive tract of *C. elegans* to investigate the role of *pha-4/FoxA* in whole organism responses to nutrients. My search for *pha-4/FoxA* regulators has lead to insights regarding how an animal responds to nutrients during aging in the adult and postembryonic development.

I have examined the role of *pha-4/FoxA* during adult lifespan extension due to loss of nutrient signaling through the Target of Rapamycin (TOR) pathway. To search for regulators of *pha-4/FoxA*, Dustin Updike performed a genetic screen to search for suppressors of the larval lethality associated with the *pha-4* mutation and discovered an AAA ATPase, ruvb-1, as a potent suppressor. I showed that the *ruvb-1* mutant phenocopies mutations in the TOR pathway suggesting that these genes have a similar function in protein biosynthesis. I found that *pha-4/FoxA* is required for extension of adult lifespan by loss of CeTOR signaling; however, only mutations in one of the predicted CeTOR targets, *rsks-1/S6K,* required *pha-4/FoxA* for lifespan regulation. These data suggest a model where TOR signaling through S6 kinase antagonizes *pha-4/FoxA* factor activity.

Secondly, I have investigated the role of *pha-4/FoxA* during L1 diapause induced by starvation. Proper developmental arrest in response to starvation has been shown to be required for survival however little is known about factors that are required during L1 diapause. I showed that *pha-4/FoxA* is required for L1 diapause survival post embryonically. I showed that levels of PHA-4 are important for starvation survival. *pha-4/FoxA* is required for initiation of development after periods of starvation and is not required for developmental arrest during starvation. We are currently using genomewide approaches to determine targets of *pha-4/FoxA* that are important for L1 diapause recovery.

This work investigates how nutrients regulate whole body responses through the GI tract. I find that *pha-4/FoxA* plays critical roles in coupling low nutrient intake to aging and development.

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CHAPTER 1

INTRODUCTION

1.1 Overview

My thesis work investigates how nutrient intake controls aging and development through the gastrointestinal (GI) tract. The GI tract is the central player in the responses of the whole organism to nutrients. I use the simple digestive tract configuration in *C. elegans* to investigate the role of the gut in the regulation of aging and postembryonic development by nutrients. I start with a full discussion of GI tract function and role in starvation and aging in mammals. This gives the reader a framework in which to discuss what is known about the *C. elegans* digestive tract. Finally I provide a discussion on the transcriptional response of the GI tract by focusing on the FoxA transcriptional regulators in both mammals and *C. elegans*.

1.2 Mammalian Gastrointestinal (GI) Tract

The mammalian gastrointestinal (GI) tract is made up of multiple organs that are required for one main function: the digestion of food. The primary digestive organs form a linear passageway for food to be taken in and broken down into nutrients for distribution to the body and removal of waste products. This group of organs consists of the mouth, esophagus, stomach, small and large intestine, rectum and anus. The hallmark of this group is that they form a hollow linear muscular tube that is lined with an inner mucosal surface. Accessory digestive organs are also required to produce and store enzymes for complete digestion. This group of organs is consists of the liver, pancreas and gallbladder. The hallmark of this group is that the organs are all secretory and have direct access into the primary digestive tract. Reviewed in (Kimball, 1994).

1.2.1 GI Tract Function

The GI tract performs four essential functions to distribute nutrients to the body from outside food sources: motility, digestion, absorption and elimination. Motility of food through the esophagus, stomach and intestines is achieved by coordination of muscle contractions (Schulze, 2006). The GI tract is the responsible for all digestion of food by both mechanical and chemical methods into disaccharides, peptides and fatty acids that can be delivered to the rest of the body for energy (Kong and Singh, 2008). The gut is lined with mucosa that can absorb nutrients directly across cell membranes into the bloodstream (Crane, 1968). Molecules that cannot be digested or absorbed need to be eliminated from the body (Bharucha, 2008). The dysfunction of any one of these processes leads to many common disorders including indigestion, peptic ulcer disease, inflammatory bowel disease and irritable bowel syndrome (Lacy and Weiser, 2006; Schubert and Peura, 2008).

Because the GI tract is a long tube into which substances enter, pass through and leave, motility through the various parts of the system at the correct time is important for all other steps of the digestive process. The act of swallowing food initiates peristalsis in which coordinated muscle contractions begin in the esophagus and push food through the GI tract (Dodds et al., 1973). The stomach has important regulated motor functions including mixing food and emptying contents to the next chamber. Mixing of stomach contents is required for complete breakdown of food (Pallotta et al., 1998; Schulze, 2006). Gastric emptying rate is important for absorption and changes with meal composition (Kong and Singh,

2008). The intestine experiences both macroscopic and microscopic physiological forces. The entire bowel walls contract to allow material to be passed from the stomach, and mucosal surface changes dramatically to achieve the appropriate volume to surface area ratio for optimal absorption (Crane, 1968; Karaus and Wienbeck, 1991).

Digestion is the act of breaking down food into single molecules. The digestion process begins the instant food enters the mouth. The mouth breaks down large pieces of food by mastication and mixing digestive enzymes produced in the salivary glands (Mackie and Pangborn, 1990; Yeh et al., 2000). This ensures that large pieces are the appropriate size and constitution to proceed through the GI tract. The bulk of digestion happens in the stomach by both mechanical and chemical means. The stomach mixes food particles and enzymes in a highly acidic environment maintained by the secretion of gastric acid (Pohl et al., 2008). Particles must be broken down into certain sizes to pass through to the intestine (Bharucha, 2008; Kong and Singh, 2008).

The majority of absorption of nutrients happens in the small intestine. The pancreas and liver produce digestive enzymes that are pumped into the duodenum (first section of small intestine) to facilitate breakdown of particles into single molecules (Wormsley and Goldberg, 1972). To maximize the surface area of absorption, the small intestine contains unique structures such as circular folds, villi and microvilli. These structures project into the lumen of the intestine and allow the direct diffusion of nutrients through the epithelium to the capillaries and into the blood stream (Crane, 1968). These projections increase the surface area of the

intestine for maximal absorption of nutrients. These projections are highly dynamic and respond to food intake status by extending and contracting (Crane, 1968). Multiple transporters facilitate the uptake of sugars, minerals, vitamins, peptides and amino acids into cells (Ferraris and Diamond, 1989).

The lower GI tract is responsible for recycling and elimination of digestion waste products. Excess debris is shed into the colon, stored in the rectum and extruded through the anus. The colon is the major site for the absorption of water and electrolytes (Turnberg, 1970). Colonic bacteria break down fibers and release nutrients that maintain a healthy colonic mucosa (Davis and Milner, 2009). The rectum stores waste until the nervous system triggers muscle activity for extrusion (Bharucha, 2008).

Although commonly known for its role in digestion, the GI tract has other important roles in the body. Because outside substances are being brought into the body, the GI tract plays a critical role in immune defense. The acidity of the stomach kills most bacteria; however, there are also many specialized cells that are produced in the intestine that recognize and destroy pathogenic bacteria (Honda and Takeda, 2009). The architecture of the intestine also is a line of defense. Many intestinal specific enzymes detoxify xenobiotics and antigens. Immunity is achieved by both blocking unwanted substances from entering the bloodstream and active removal of reactive immune cells (Turner, 2009).

1.2.2 Regulation of Energy Homeostasis by the GI Tract

The GI tract is a central sensor and regulator of energy homeostasis in the body. Energy intake by either feeding or storage mobilization must equal the amount of energy expenditure. The GI tract dynamically responds to different dietary conditions by regulating feeding and lipid mobilization. This is accomplished by relaying information about energy intake from the GI tract to the central nervous system (CNS).

The GI tract works in unison with the rest of the body through the CNS. The organs of the digestive system have their own network of nerves called the enteric nervous system. These nerves run along the entire length of the GI tract from the esophagus to the anus (Gershon, 1981). They are required for coordination of muscle contractions and secretions during digestion (Altaf and Sood, 2008). The autonomic nervous system is composed of nerves that run from various digestion system organs to the central nervous system (Kimball, 1994). Communication between the GI tract and the CNS are required to regulate feeding behaviors (Magni et al., 2009). Extrinsic cues such as olfaction are also transmitted through the CNS to activate digestive organs (Powley, 2000).

The GI tract plays an important role in energy homeostasis by controlling organismal behavior in response to food intake. There are two main signals, satiation and adiposity, that work with the CNS to regulate feeding (Karra and Batterham). Satiation signals tell the body about meal size or calorie intake (Brobeck, 1975). As levels of nutrients rise, many signals are sent out to the CNS to reduce feeding and movement through GI tract. These signals are in the form of

peptides and are secreted from the intestine and pancreas (Woods and Gibbs, 1989). Intestinal peptides include peptide tyrosine-tyrosine (PYY), oxyntomodulin (OXM), glucagon like peptide-1 (GLP-1), cholecystokinin (CCK) and apolipoprotein A-IV that are important in the reduction of food intake, energy expenditure, gut motility and absorption (Brubaker, 1991; Green et al., 1980; Karra and Batterham; Smith and Gibbs, 1985). The pancreas also releases pancreatic polypeptide (PP), amylin, insulin and glucagon with differential control of gastric motility and glucose homeostasis (Chaudhri et al., 2008). Only one peptide has been found to stimulate feeding. Ghrelin is secreted from the stomach and small intestine and is a meal initiator (Cummings, 2006).

Adiposity signals, such as insulin and leptin, are released in proportion to amounts of adipose tissue and signal directly to the CNS to tell the body about how much energy is already stored in the body. Insulin is a hormone secreted from the pancreatic B cells and when infused into the CNS causes decreased food intake (Woods et al., 1979). Another food intake regulator hormone, leptin is secreted directly from adipocytes (Porte et al., 2002). The circulating amounts of these hormones are directly proportional to the amount of fat in the body. Once in the bloodstream, these molecules can pass through the blood-brain barrier and interact directly with receptors in the brain to modulate behavior (Woods, 2009). High levels reduce energy intake by decreased food reward and motivation to feed (Figlewicz and Benoit, 2009). In many cases of obesity, the CNS is resistant to these signals and feeding is not coupled to the amount of energy already stored in the body (Israel et al., 1993; Owen et al., 1983). The interaction of these two signals

gives the organism a read out of how much energy intake is needed and how much energy storage must be released for overall energy balance.

1.2.3 Starvation Response

The morphology and motility of the digestive organs change in response to feeding state. The interdigestive phase, although lacking the massive up regulation of secretion and motility seen in the digestive phase, has distinct patterns of muscle contractions and secretions that are required for optimal GI tract function. During fasting the stomach continues to contract and move contents to the small intestine (Schindlbeck et al., 1989). The composition of gastric content during fasting is highly acidic and requires secretions to sustain buffer capacity and osmolarity (Kong and Singh, 2008; Malagelada et al., 1976). Longer periods of fasting cause the intestinal mucosa to become atrophic by shortening of villi and decreases digestive enzyme production (Dunel-Erb et al., 2001; Habold et al., 2007; Inoue et al., 1993).

During states of very low energy intake, mobilization of lipids and glucose metabolism programs are activated through the GI tract. The pancreas releases glucagon, which promotes gluconeogenesis in the liver (Gerich, 1981). The liver is the central controller for gluconeogenesis and ketogenesis, which maintains glucose or ketone levels, respectively, in the circulation to be used by tissues that cannot breakdown lipids for energy (Owen et al., 1969; Wahren and Ekberg, 2007). In many tissues, ß-oxidation and autophagy are activated for nutrient breakdown (He and Klionsky, 2009; Wolfrum et al., 2004).

Chronic malnutrition inhibits postembryonic development. Postnatal development is tightly regulated by food intake (Henning, 1981). Reproductive development has been shown to be delayed by malnutrition (Martos-Moreno et al., 2009). It is unclear how nutrients control whole organism responses.

1.2.4 Aging

One of the hallmarks of the older adult is the degeneration of GI tract function. The GI tract shows many changes during aging including decreased appetite and motility (Parker and Chapman, 2004). Gastric motility and emptying are slowed and may play a major role in reduced food intake (Clarkston et al., 1997; Horowitz et al., 1984; Rayner et al., 2000). Hunger-satiety signals are altered as well as extrinsic control of feeding by taste and smell (Mathey et al., 2001; Morley, 1997). Reduced food intake could be due to increase of CCK and decrease of ghrelin but other peptides show normal levels and responses (Rigamonti et al., 2002; Sturm et al., 2003).

Interestingly, aging is also modulated by dietary restriction (DR) through the GI tract. DR is the reduction of food intake with optimal nutrition. This method requires individuals to consume all necessary nutrients while decreasing the amount of total calories. The first description of this method was performed on rats by McCay and others in 1935 and described the first instance of DR correlating with a longer life. DR by different methods produces similar effects on lifespan (Goodrick et al., 1990). DR has been shown to be the only treatment that increases lifespan by 30-60% across almost all organisms tested including yeast, flies, worms and rodents

(Fontana and Klein, 2007). Studies have shown that DR slows progressive changes that occur during aging and reduces age related occurrences of cancer, cardiovascular disease and immune deficiency (Bordone and Guarente, 2005). Although DR is the most potent method of lifespan extension across organisms, little is known about the mechanisms that are required for regulation of aging.

1.3 *C. elegans* Digestive Tract

An outstanding question from mammalian research is how the gut acts as a central regulator of whole organism responses to different environmental conditions. Research of the GI tract and how it responds dynamically at the whole animal level is difficult in higher organisms. The digestive system in mammals is extremely complex with many organs required for multiple functions. Also, energy balance is achieved through regulation of feeding behavior, control of metabolic rate, efficiency of digestion and absorption, fat storage and energy expenditure. Although much is known about individual organs, little is known about how outside stimuli, such as caloric restriction or starvation, causes coordinated responses across the whole organism through the gut. *C. elegans* have a very simple gut composed of a foregut, midgut and hindgut (www.wormbase.org). These organs perform all the functions necessary for digestion, motility, absorption and elimination. Ease of genetic manipulation and visualization of internal organs makes *C. elegans* a powerful tool to investigate the complexity of energy balance.

1.3.1 Function of the Gut in *C. elegans*

The foregut is responsible for the intake and breakdown of food and progression of material into the midgut. *C. elegans* are considered filter feeders. Food in the form of *E. coli* is pumped into the body by the pharynx, a bilobed tube that functions as a two chambered pump (Albertson and Thomson, 1976; Avery, 1993; Avery and Shtonda, 2003). Bacteria and liquid are pumped in by contractions of the muscles. Relaxation of the pharynx causes liquid to be expelled while bacteria are trapped by cuticular protrusions (Fang-Yen et al., 2009). Only particles of a certain size can be pumped into the pharynx and proceed through the gut. Once trapped, the bacteria are forced through the grinder, which is composed of cuticular protrusions that form interlocking teeth (Albertson and Thomson, 1976). This disrupts the cell membranes and releases the bacterial contents. Defects in grinding cause intake of intact bacterial cells of which cannot be digested in the midgut (You et al., 2006).

The pharynx is composed of multiple cell types with specific functions (Albertson and Thomson, 1976). Gland cells have processes that allow secretions to be pumped directly into the pharyngeal lumen (Nelson et al., 1983). The makeup of the secretions is unknown but genes expressed exclusively in the glands play a role in food intake and molting (Mango, 2007; Ohmachi et al., 1999). Valve cells form a barrier between the foregut and hindgut so that material cannot go backwards. Neurons control muscle contractions and pumping rates (Avery, 1993; Avery and Horvitz, 1989; Raizen and Avery, 1994; Raizen et al., 1995). These neurons couple

environmental conditions to pumping. Defects in pharyngeal neurons cause severe feeding defects (Avery, 1993).

The midgut is composed of only one cell type, the enterocyte, which composes the entire intestine (McGhee, 2007). Cells are placed side by side with a lumen through the entire length. Each cell has an apical domain that is covered with a brush border very similar to mammals. This brush border contains microvilli that extend into the lumen which by EM look to be incased in unstructured glycoprotein (Lehane, 1997). This covering is thought to be play an important role in protection from damage and pathogens, creates a filter for nutrients and is a scaffold for digestive enzymes (Borgonie et al., 1995). These cells also contain intestine specific organelles such as lysosome-related gut granules and ß-oxidation peroxisomes (Clokey and Jacobson, 1986; Hermann et al., 2005; Togo et al., 2000; Yokota et al., 2002).

Digestion and adsorption takes place in the intestine and depends heavily on the actions of digestive enzymes and nutrient transporters. The intestinal cells express hydrolases, proteases, peptidases and lipases much like the mammalian intestine (Brooks et al., 2003; Jo et al., 2009; Joshua, 2001; Laurent et al., 2001; McGhee, 2007). However, unlike mammals, nutrients only spend very short amounts of time in the intestinal lumen so active transport of nutrients and endocytosis is required for nutrient uptake (Meissner et al., 2004; Nehrke, 2003; Sato et al., 2005). Nutrients are taken up into the cells from the lumen by various transporters, including dipeptide, ion, and nucleoside (Appleford et al., 2004; Meissner et al., 2004; Nehrke, 2003; Sherman et al., 2005; Teramoto et al., 2005).

Acidification of the intestinal lumen has been shown to be required for adsorption of nutrients. The lumen is acidified by vacuolar ATPases and controlled by sodium-hydrogen exchangers such as *nhx-2* (Allman, 2009; Nehkre, 2003). Destroying the ability of the intestine to absorb nutrients in any manner leads to metabolic defects including growth defects, decreased reproduction, increased fat storage and delay in aging (Allman, 2009; Nehkre, 2003; Meissner et al., 2004; Spanier et al., 2009).

The hindgut is composed of several cell types and functions in the elimination of waste. The intestine is connected to the rectum by a rectal valve. Defecation consistently occurs every 50 seconds and requires enteric muscles and neurons that coordinate contractions across the whole intestine (Thomas, 1990). These contractions are responsible for movement of nutrients through the intestine as well as elimination of waste (www.wormatlas.org).

The intestine like higher organisms is the major site of protection against foreign substances. Many genes involved in detoxification such as cytochrome P450 enzymes and P-glycoproteins are highly expressed in the intestine (McGhee et al., 2007). Pathogenic bacteria are highly invasive and can colonize and kill worms quickly (Darby, 2005). The GI tract has a very complex immune response to foreign bacteria (McGhee, 2007).

1.3.2 Regulation of Energy Homeostasis by the Gut in *C. elegans*

Similar to mammals, *C. elegans* have a very complex regulation of feeding behavior through the nervous system. The behavior of *C. elegans* changes whether they are on food or away from food. When taken off of food they transition from a

local search behavior to traveling (Gray et al., 2005; Wakabayashi et al., 2004). These adaptations to food availability require chemosensory neurons (Sengupta et al., 1993). Serotonin, well known for its role as a neurotransmitter in the mammalian GI tract to modulate behavior, is absolutely required for these changes in foraging behaviors (Bargmann, 2006).

Neuronal signaling regulates fat homeostasis. Defects in chemosensory neurons and signaling components have altered levels of stored lipids in the intestine (Ashrafi et al., 2003; Cohen et al., 2009). Chemosensory neurons express peptides that activate signaling in multiple tissues including the intestine. *C. elegans* use many signaling pathways similar to mammals to regulate fat homeostasis including RFamide, muscarinic, serotonin, insulin and TGF-ß (Cohen et al., 2009; Greer et al., 2008; Srinivasan et al., 2008).

Worms also adjust fat metabolism programs through mechanisms similar to mammalian pathways. One of the many nuclear hormone receptors found in worms is *nhr-49*, similar to mammalian PPAR, which controls ß-oxidation and fatty acid desaturation (Taubert et al., 2006; Van Gilst et al., 2005b). Sterol response element binding protein (SREBP) homolog, *lpd-1*, also is required for fatty acid elongation and fat storage (Ashrafi, 2006; McKay et al., 2003; Nomura et al., 2009; Yang et al., 2006).

1.3.3 Starvation Response in *C. elegans*

Similar to mammals, worms respond to energy demand by changes in gut motility and morphology. Pharynx function is tightly regulated by the nervous

system. Pumping in the pharynx is very sensitive to external conditions. Food status and quality will change rates of pumping (Avery and Horvitz, 1990; Shtonda and Avery, 2006; You et al., 2006). Changes in pumping rates are driven by biogenic amine neurotransmitters including octopamine, tyramine and serotonin (Chase and Koelle, 2007). Acetylcholine also controls pharynx muscle through muscarinic signaling (You et al., 2006).

Starvation causes major changes in the *C. elegans* gut very similar to mammals. In the first few hours of starvation pumping and food seeking is increased (Avery and Horvitz, 1990). However long term starvation causes shut down of feeding and relaxation of pharynx muscle (You et al., 2006). During starvation pharyngeal muscle is sensitized to food inputs and pumping is stimulated by much lower concentrations of food than in the fed state (You et al., 2006). Starvation, limitation of nutrients and mutations in nutrient signaling all cause delays in development, growth and aging in the worm.

Classically, starvation in *C. elegans* has been studied in dauer larvae. After embryogenesis, worms proceed through four larval stages until they become reproductive adults. Starvation at the L1 to L2 stage transition leads to an alternative larval stage, called dauer, that is extremely long-lived and stress resistant (Cassada and Russell, 1975). Dauer larva completely shut down feeding. The pharynx is constricted and pumping is stopped and the intestine thins and microvilli are all but absent (Riddle et al., 1981; Vowels and Thomas, 1992). Dauers use the glyoxylate pathway to convert lipids into carbohydrates for energy (Wadsworth and Riddle, 1989). Four signaling pathways have been shown to

regulate dauer: guanyl cyclase, TGF-ß, insulin and steroid hormone (Antebi et al., 2000; Birnby et al., 2000; Gems et al., 1998; Motola et al., 2006; Patterson et al., 1997; Ren et al., 1996). Environmental sensing through chemosensory neurons is important for dauer arrest (Riddle et al., 1981).

Starvation causes growth arrest at multiple stages in postembryonic development including L1 diapause, dauer and adult reproductive diapause. Worms exposed to starvation at any stage will arrest growth. Cessation of growth and/or reproduction is absolutely required to survive periods without food. Mutants that fail to stop cell divisions during starvation either during L1 arrest, such as *daf-18*/PTEN and *daf-16*/FoxO, or during reproductive diapause, such as *nhr-49*, cannot survive periods of starvation (Angelo and Van Gilst, 2009; Baugh and Sternberg, 2006; Fukuyama et al., 2006).

Starvation survival is modulated through various responses. Signaling through chemosensory neurons is required for sensing of food intake and coordinating responses throughout the body through insulin and MAP kinase signaling (Kang and Avery, 2009; Lee and Ashrafi, 2008; You et al., 2006). Recycling and reuse of cellular components is important during starvation and defects in protein repair and autophagy have decreased starvation survival (Gomez et al., 2007; Kang and Avery, 2009). Regulation of lipid metabolism through transcriptional activation by nuclear hormone signaling is also a component of the starvation response (Brock et al., 2006; Van Gilst et al., 2005a). These data show that there is important communication between nutrient sensing and metabolism,

but how many of these responses are controlled at the transcriptional level is still unclear.

1.3.4 Regulation of Aging

Aging causes major changes in the gut of *C. elegans*. Several characteristics have been described that track with age. Pumping of the pharynx decreases and eventually stops completely a few days before death (Huang et al., 2004). The intestine accumulates "age pigments" which are thought to be the accumulation of damaged proteins (Gerstbrein et al., 2005). The defecation program becomes slowed and less frequent (Bolanowski et al., 1981).

Aging is modulated by nutrient intake. Starvation initiated during postembryonic development or reproduction causes severe growth arrest. However, if food is taken away from postreproductive adults, lifespan is extended (Kaeberlein et al., 2006; Lee et al., 2006). Starvation causes increased stress resistance and decreased proteotoxicity that may be protecting animals from the effects of aging (Steinkraus et al., 2008). However, the mechanism of the effect of complete removal of food on lifespan is not known and it is unclear if starvation affects lifespan in a similar manner in higher organisms.

C. elegans have become a premier model to study DR and its effects on lifespan due to their short lifespan and ease of genetic manipulation. The most common method to induce DR in worms is by genetic mutation or by restricting access to food. Certain genetic mutants such as *eat-2* have defects in the muscles or nervous system of the pharynx causing a decrease in food intake throughout the

entire life and an increase of 26-42% in lifespan (Lakowski and Hekimi, 1998). Another way to limit food intake is by growing worms in limited concentrations of bacteria. This can be done in liquid culture (bacterial dietary restriction (bDR)) (Panowski et al., 2007) or solid culture (solid dietary restriction (sDR)) (Greer et al., 2007). These methods provide limited access to food at all times and increase lifespan from 28-60% (Bishop and Guarente, 2007b; Panowski et al., 2007). This strategy is used to test DR effects during adulthood and avoid any developmental effects. This method is attractive because the amount of food intake can be tightly controlled and can be used to restrict food for short amounts of time.

Dietary restriction modulates starvation survival through the neurons and gut. Of the transcription factors that have been identified to be important in DR response all are found in the neurons and intestine. These factors include *daf-16/FoxA, hsf-1/HSF, pha-4/FoxA* and *skn-1/NRF* (Bishop and Guarente, 2007b; Greer et al., 2007; Panowski et al., 2007; Steinkraus et al., 2008). Their common expression in both tissues suggests an intimate role between neurons and the intestine that controls DR response. However, *skn-1* was shown to be required in the neurons only for lifespan modulation (Bishop and Guarente, 2007a) suggesting that in some cases lifespan can be modulated directly through neuronal signaling.

1.4 Transcriptional Response of the GI Tract

One of the most striking results from genome-wide gene expression studies in worms is that there is not only an enrichment of metabolic genes but also transcription factors in the intestine (McGhee et al., 2007). Many of the same

transcriptional programs that regulate lipid metabolism and homeostasis in mammals also are functional in worms. There is much similarity between mammals and worms at the transcriptional level of GI tract regulation of metabolism.

FoxA factors, members of the forkhead transcription family, are well known transcriptional regulators of gut development and function. FoxA factors were first shown to be required for foregut development across many organisms (Friedman and Kaestner, 2006). Later it was shown that FoxA homologs were necessary for development and maintenance of gut function in the adult as well.

1.4.1 FoxA Factor Role in Development

FoxA factors were first discovered as master regulators of gut development. In invertebrates, there is one FoxA that is required for development of the foregut (Horner et al., 1998; Mango et al., 1994). In mice, two of the three FoxA homologs, FoxA1 and FoxA2, are required for specification of the gut in early development and function of the digestive system after birth (Burtscher and Lickert, 2009). FoxA factors in mice have partial redundancy, which are unmasked in multiple FoxA knockouts. Knockout of FoxA1 and FoxA2 causes massive development phenotypes including loss of specification of the liver and pancreas (Gao et al., 2008).

FoxA factors are required in other tissues beyond the GI tract. FoxA2 and FoxA1 are required for dopaminegenic neuron specification and function (Kittappa et al., 2007). FoxA3 has been shown to be important in germ cell maintenance (Behr et al., 2007). FoxA1 and FoxA2 are required together for proliferation, differentiation and branching in the fetal lung (Tichelaar et al., 1999; Wan et al.,

2005). Prostate development shows early expression of FoxA1/A2 and requires them for hormone specific gene expression (Mirosevich et al., 2005; Yu et al., 2005). *pha-4/FoxA* is required for gonad development in *C. elegans* (Updike and Mango, 2007). FoxA factors also play important roles in the development and maintenance of the kidney, lung and mammary tissue (Friedman and Kaestner, 2006).

FoxA factors are required throughout the entirety of development for activation of multiple steps of organ development. Liver specific embryonic knockouts of FoxA1 and A2 at later developmental time points after differentiation show defects in formation of later structures such as duct formation (Li et al., 2009). These factors are also required for both differentiation and specification of intestinal cells into specialized secretory cell types such as L cells, D cells and goblet cells (Ye and Kaestner, 2009).

1.4.2 FoxA Factor Role in Disease

FoxA factors play a role in many cancers. The majority of breast cancers are estrogen dependent and FoxA1 plays an important role in estrogen receptor (ER) activation of many targets that modulate breast cancer growth and survival. FoxA1 modulates binding of ER to many of its targets (Carroll et al., 2005). Loss of FoxA1 in breast cancers correlates with lower survival, and higher level of FoxA1 is correlated with lower grade cancers, smaller tumor size and better survival (Albergaria et al., 2009; Badve et al., 2007; Habashy et al., 2008; Thorat et al., 2008; Wolf et al., 2007). A recent study has shown that FoxA1 tracks with luminal cells of the mammary gland therefore marking more differentiated cells and thus less

aggressive cancers (Yamaguchi et al., 2008). Similarly, FoxA1 is correlated with prostate cancer and androgen receptor (AR) (van der Heul-Nieuwenhuijsen et al., 2009). FoxA interacts with AR and regulates many of the growth and differentiation pathways that are misregulated in prostate cancer (Lee et al., 2008). Other cancers, such as anaplastic thyroid carcinoma, have shown high FoxA1 expression (Nucera et al., 2009). FoxA factors may be an important generally for regulation of growth and survival during the progression of cancer.

During cancer progression, cells lose their ability to function correctly, which leads to death and damage. FoxA factors have been implicated in regulating cell death, autophagy and cell survival (Lehmann, 2008; Taubert et al., 2008). Heat shock factor proteins are induced in cells in response to heat stress and promote survival through protein maintenance. HSP72 correlates with severity of tumors and expression is modulated by levels of FoxA1 in MCF-7 cells (Song et al., 2009). Cell cycle regulators are also important in the growth and progression of cancer. FoxA1 causes cell cycle arrest by activating the cell cycle inhibitor p27kip1 (Wolf et al., 2007)

FoxA factors are required for the transcriptional control of glucose homeostasis (Shen et al., 2001; Zhang et al., 2005), which make them good candidates to be important in the development of diabetes. Several groups have found FoxA alleles that track with maturity onset diabetes of the young (MODY) and type II diabetes (Navas et al., 2000; Tabassum et al., 2008; Yamada et al., 2000; Zhu et al., 2000). Specifically in type II diabetes, one group found two different FoxA2 alleles that have opposing effects. A mutation in the 3'UTR has a protective effect

against diabetes whereas another mutation in intron 1 tracks with obesity and onset of diabetes (Tabassum et al., 2008). These mutations have not been functionally characterized thus the mechanism of FoxA in diabetes is still very unclear.

1.4.3 FoxA Factor Regulation of Metabolism

Although well known for developmental roles, the FoxA factors are required for transcriptional responses in the GI tract. In mice all FoxA factors are important for maintenance of glucose homeostasis in response to fasting (Shen et al., 2001; Zhang et al., 2005). In *C. elegans pha-4/FoxA* is required for organismal responses to nutrient intake (Ao et al., 2004; Panowski et al., 2007; Sheaffer et al., 2008).

Glucose metabolism is tightly regulated by food intake. When glucose levels are limited in the blood, gluconeogenesis is up regulated in the liver to generate glucose from noncarbohydrate carbon substances. The pathway is a series of enzymatic reactions that are regulated by the amounts of rate-limiting enzymes such as Phosphoenolpyruvate Carboxykinase (PEPCK) and Glucose-6-phosphatase (G6P) (Exton, 1972). The gluconeogenic program requires hormone regulated transcriptional activators such as the glucocorticoid receptor (GR) and cAMP response element binding (CREB) to coordinate expression of targets (Imai et al., 1993). PEPCK is induced by starvation and requires FoxA2 for induction (Wolfrum et al., 2003; Wolfrum and Stoffel, 2006). Other genes sensitive to hormonal regulation during fasting, such as tyrosine aminotransferase (TAT) and insulin growth factor binding protein-1 (IGFBP-1), also required FoxA2 for induction of expression (Wolfrum et al., 2003; Wolfrum and Stoffel, 2006). FoxA2 was shown to

be required for binding of GR and CREB to these targets during starvation (Zhang et al., 2005).

pha-4/FoxA has also been shown to be important in nuclear hormone signaling in worms. *daf-12* encodes a NHR that is known for its role in integration of environmental sensing by insulin and TGF-ß signaling into regulation of transcription of targets important in metabolism, dauer diapause and lifespan (Antebi, 2006). A *daf-12* binding motif in promoters of *pha-4/FoxA* targets was found to be required for the differential expression of genes in the pharyngeal muscle during dauer. *pha-4* was shown to be required for morphologic changes in the pharynx specifically during exit from dauer (Ao et al., 2004). These data suggest that *pha-4* is required for transcriptional activation in the pharynx and may be important for the integration of multiple environmental signals with other factors, such as *daf-12,* to modulate expression of genes important for dauer recovery.

Across organisms, FoxA factors have been shown to be required for the proper maintenance of cell function during the aging process. FoxA2 heterozygous mice show progressive loss of dopamine neurons and motor behavior in old age (Kittappa et al., 2007). FoxA2 RNA levels decrease as the liver ages and differential binding to ApoA1 promoter causes decreased transcription in 6-month-old mice compared to newborns (Nakamura et al., 1999). *pha-4/FoxA* is required for normal lifespan in *C. elegans* (Panowski et al., 2007; Sheaffer et al., 2008). Nutrient signaling during aging regulates *pha-4/FoxA* activity. Increased lifespan due to dietary restriction and reduced Target of Rapamycin (TOR) signaling requires *pha-4/FoxA* (Carrano et al., 2009; Greer and Brunet, 2009; Panowski et al., 2007;

Sheaffer et al., 2008). The nature of *pha-4* activity regulation by nutrient signaling is still unclear and will be discussed in the next sections.

1.4.4 FoxA Factor Mechanism of Action

Studies of FoxA factors in development, disease and metabolism have given us insight on how these factors modulate transcription. FoxA factors are required for both activation and repression of transcription (Li et al., 2009). FoxA factors are considered pioneer transcription factors that bind DNA and open up chromatin to allow accessibility for other factors. FoxA factors function by binding within regulatory sites of targets. FoxA sites can be found in close (1kb) and long (500kb) range from start site of targets (Carroll et al., 2005; Wederell et al., 2008). FoxA factors have different binding affinities for different amino acid sequences, and this is important for expression of targets (Gaudet and Mango, 2002). In some cases, interactions with other transcriptional regulators are important for full activation and tissue specificity of FoxA targets.

FoxA factors have been shown to have high affinity for DNA and interact directly with nucleosomes. The wing structures in the FoxA factors have been shown to have very close contacts with the minor groove of DNA (Cirillo and Zaret, 2007) and bind directly to nucleosome cores (Chaya et al., 2001; Cirillo et al., 1998). FoxA factors have high nonspecific binding to both nucleosomes and free DNA. This causes nuclear mobility that is much slower than multiple other transcription factors and most closely resembles H1 (Sekiya et al., 2009).

FoxA factors, because of their high affinity for DNA, can bind chromatin directly and open it up for transcription. FoxA1 can bind DNA that has been assembled with nucleosomes in vitro and cause periodic nucleosome spacing (Cirillo et al., 2002). In multiple cell lines FoxA1 binding is correlated with closed chromatin configuration suggesting that FoxA1 has the ability to associate with highly compacted DNA (Eeckhoute et al., 2009). FoxA1 causes nucleosome rearrangement during transcriptional activation of promoters. This has been nicely demonstrated using the mouse mammary tumor virus (MMTV) promoter. Full transcriptional activation of MMTV causes specific nucleosome repositioning which has been well characterized in tissue culture (Richard-Foy and Hager, 1987). FoxA1 alone was shown *in vivo* to fully activate the nucleosome repositioning (Belikov et al., 2009). However, most of this work has been shown in artificial systems, and the role of FoxA factors in nucleosome positioning and activation of promoters *in vivo* is still unclear.

Studies have shown *in vivo* that FoxA factors do have roles in establishing transcriptional competence in chromatin. In mice, FoxA1 and FoxA2 are required to establish competence of foregut endoderm (Lee et al., 2005). During early liver specification, FoxA1 binds the serum albumin enhancer and activates the promoter (Gualdi et al., 1996). This is also seen in organ development where *pha-4/foxA* is required to activate chromatin by recruiting H2A.Z and activating pharynx genes in *C. elegans* (Updike and Mango, 2006).

Another important aspect of activation of promoters by FoxA factors is binding site affinity. This has been most well characterized in *C. elegans* where *pha-*

4/foxA has been shown to bind different DNA sequences with different affinity. Temporal activation of targets during development is achieved by binding site affinity. Promoters with high affinity sites are generally activated early in development compared to promoters with low affinity sites (Gaudet and Mango, 2002). Binding site affinity may also direct tissue specific expression. In adult mice there is a correlation between genes with FoxA factor consensus sites and expression level in two or more tissues (Tuteja et al., 2008). Recently a mutation in the consensus site of FoxA1 on human chromosome 8q24 was found to be important in activation of an AR enhancer implicated in prostate cancer (Jia et al., 2009). These data suggest that affinity for DNA binding sites may play a major role in FoxA regulation of targets both during development and disease.

The regulation of FoxA target expression is further fine-tuned by cis acting elements within regulatory sites. Investigation of promoters of many *pha-4/FoxA* targets activated at different times in development show cis-acting sequences that are enhancers or repressors of gene expression (Gaudet et al., 2004). Binding of FoxA2 must be maintained throughout development to keep promoters competent for binding of factors. Even in later phases of liver development FoxA2 is required for GR binding to down regulate IL-6 to allow correct development of duct formation (Li et al., 2009). In mice FoxA factors have been shown to be important for binding of well-known transcriptional regulators that are hormone dependent in multiple contexts including AR, ER and glucocorticoid receptor (GR) (Cao et al., 2007; Holmqvist et al., 2005). FoxA factors, found in enhancer regions distal to target genes, show the same mechanism. FoxA1 occupies an upstream enhancer of

serum albumin gene and is required for binding other factors such as Gata-4, NF-1 and C/EBP that are required for expression (Bossard and Zaret, 2000; Cirillo et al., 2002; Gualdi et al., 1996). Some targets like Pdx-1 require FoxA2 to bind a distal enhancer as well as the proximal promoter to activate expression (Gao et al., 2008). Combinations of FoxA factors and other transcriptional activators provide extremely tight control of gene expression.

Although there are many data suggesting that FoxA interacts with cofactors, it is still unclear what factors have direct interactions. PGC-1 beta, a transcriptional activator of metabolic genes, was shown to interact directly with FoxA2 by co-immunoprecipitation. Even though FoxA2 was not absolutely required for expression of target genes this interaction with PGC-1 beta led to a more robust activation of ß-oxidation genes such as CPT1, Mcad and Vlcad (Wolfrum and Stoffel, 2006). A kinase, DNA-PK, has also been suggested to be important for the regulation of transcriptional activity of FoxA2 in the context of the ApoA1 enhancer. However, phosphorylation of FoxA2 by DNA-PK has no effect on the ability of FoxA2 to bind DNA and may be required for general activation of transcription (Nock et al., 2009).

FoxA factors are found in many tissues with very different gene expression profiles. There is evidence to suggest that tissue specificity of FoxA factors may be due to specific cell type interactions with other co-factors and/or chromatin marks. FoxA factors are differentially recruited to cell specific targets. Initially prostate cancer cells are dependent on AR for growth but lose their dependency as the growth continues. FoxA1 has been shown to be required for AR binding and activation of targets but is also required for activation of targets that are AR

independent (Wang et al., 2009). Comparison of breast cancer (MCF7) and prostate cancer (LNCaP) cell lines showed that the majority of FoxA1 sites are lineage specific. Correlation of H3K4 methylation and FoxA1 binding may cause differential recruitment of cofactors to sites in LNCaP cells (Lupien et al., 2008). However there are many instances where FoxA1 is found on similar sites with nearby cofactors such as ER and AR in a nonlineage specific manner. Although these FoxA sites are similar in two or more cell types, inactive targets in one cell type often are found to be active in other cell types suggesting that tissue specific cofactors are required for transcriptional activation of FoxA1 targets (Eeckhoute et al., 2009).

1.4.5 FoxA Factor Regulation by Nutrient Intake

FoxA factors are required for metabolic transcription events during different dietary regiments. These data suggest that nutrient signaling plays a role in regulation of FoxA activity. Both Insulin and Target of Rapamycin (TOR) signaling have been implicated in the regulation of FoxA activity. Insulin is a hormone that is released by the pancreas in response to high levels of nutrients. It activates the uptake of glucose and energy storage as well as cell growth and survival (Hietakangas and Cohen, 2009). The TOR pathway is also activated by nutrients and is a key activator of translation and growth as well as cell survival. The central player is TOR kinase that acts in a complex to regulate many downstream targets (Hietakangas and Cohen, 2009).

Insulin initiates a signaling pathway that activates a cascade of kinases including Akt-1 and Akt-2 that control various transcription factors including

forkhead family members such as FoxO (Hietakangas and Cohen, 2009). Phosphorylation of FoxO causes nuclear exclusion and inactivation of transcription. Activation of FoxO targets is regulated by nuclear localization (Baumeister et al., 2006; Huang and Tindall, 2007). Because members of the forkhead transcription family are so similar, there has been speculation that FoxA could be phosphorylated and excluded from the nucleus much like FoxO. Furthermore, addition of insulin to Hep2 cells causes decreased activity of various FoxA targets such as PEPCK and Igfbp-1 (Wolfrum et al., 2003; Wolfrum and Stoffel, 2006).

FoxA2 localization and activity may be regulated directly by Akt2 *in vitro*; however, there is much debate on whether this mechanism is important *in vivo*. *In vitro*, FoxA2 contains a site that can be phosphorylated by Akt2. Addition of insulin or Akt phosphorylation leads to nuclear exclusion and inactivation of FoxA2 in Hep2 cells (Wolfrum et al., 2003). Of all the FoxA factors, FoxA2 has only been shown to have this Akt specific site and translocation ability by a nuclear export sequence (Howell and Stoffel, 2009; Wolfrum et al., 2003). Multiple experiments done *in vivo* have shown that FoxA2 is localized in the nucleus in the liver of starved mice (Wolfrum et al., 2004) (Zhang, Rubins et al. 2005). However, in the fed state, there are opposing data on the effect of food and insulin signaling on FoxA2 localization. One group has shown that feeding causes nuclear exclusion of FoxA2 suggesting that transcriptional activity is regulated solely by localization (Wolfrum et al., 2004). This same group has shown that mouse models of insulin resistance have constitutive cytoplasmic FoxA2 localization in the liver no matter what feeding state, suggesting that insulin signaling plays an important role in FoxA2 localization

in vivo (Wolfrum et al., 2004). However, another group has seen FoxA2 constitutively in the nucleus of the liver using similar conditions mentioned above such as fed vs. fasted and insulin resistant mouse models (Zhang et al., 2005).

Whether FoxA2 transcriptional activity is regulated by nuclear translocation is still unclear. Many FoxA2 metabolic targets show constitutive binding to promoters including Igfbp-1, PEPCK and TAT suggesting that even if FoxA is being shuttled out of the nucleus, there is no effect on its role in binding of target promoters (Wolfrum et al., 2003; Zhang et al., 2005). These data suggest a model of action by insulin that causes nuclear exclusion and inactivation of FoxA but this has not yet been proven.

Phosphorylation of FoxA2 is functional in modulating insulin resistance. Insulin resistance, diabetes and obesity are known to have defects in lipid metabolism and serum lipid abnormalities. Overexpression of the unphosphorylatable form of FoxA2 in the livers of insulin resistant mice restores lipid homeostasis and increase of serum high-density lipids (HDL) (Wolfrum et al., 2004; Wolfrum et al., 2008; Wolfrum and Stoffel, 2006). Mice with reduced levels of FoxA2 show compromised lipid metabolism (Wolfrum et al., 2004; Wolfrum et al., 2008). These data suggest that FoxA2 is inactivated by phosphorylation and an unmodified form of FoxA2 can function in the activation of targets.

FoxA factors are also modulated by dietary restriction. In mice, FoxA factors are negatively regulated by protein and amino acids postembryonically. Rats fed a protein free diet showed elevated RNA levels of FoxA3 (Imae et al., 2000). Another study showed adult mice fed amino acid free diets showed increased mRNA levels of

both FoxA2 and FoxA3 in the liver (Su et al., 2009). It would be interesting to test FoxA levels and transcriptional activity in mice exposed to DR.

In *C. elegans, pha-4/FoxA* is required for DR lifespan extension. Using *eat-2* mutants or exposing worms to bacterial DR increases lifespan (Lakowski and Hekimi, 1998). This increase in lifespan requires activity of *pha-4/FoxA* (Greer and Brunet, 2009; Panowski et al., 2007). Reduced *pha-4* has no effect on lifespan in other long-lived mutants including *daf-2/InsR* (Panowski et al., 2007; Sheaffer et al., 2008). *pha-4/FoxA* was shown to be required for lifespan extension by reduced TOR signaling (Sheaffer et al., 2008). These data show that *pha-4/FoxA* is a genetic interactor with TOR signaling, not insulin.

The mechanism of transcriptional activation of *pha-4/FoxA* during DR is still unclear in *C. elegans*. PHA-4 protein localization in the nucleus and overall levels do not change (Panowski et al., 2007; Sheaffer et al., 2008). Superoxide dismustases, *sod-1, sod-2, sod-4* and *sod-5*, were shown to be targets of *pha-4* during DR (Panowski et al., 2007). Only *sod-2* has been shown to be important in lifespan extension, and it is unclear if any of the SODs play a role in DR (Van Raamsdonk and Hekimi, 2009) .

1.4.6 Role of *pha-4*/FoxA in the Response of the GI Tract to Food

There are still outstanding questions about the role of FoxA factors in the regulation of transcriptional response of the GI tract. This work addresses two major questions in the field: the regulation of *pha-4/FoxA* by nutrients and how *pha-4/FoxA* modulates the response of the GI tract to different nutrient states. All

studies of FoxA factors in mice have been done in specific GI tract organs. Because of the simple configuration of the digestive tract, I have used *C. elegans* to look at the requirement of *pha-4/FoxA* during dietary restriction regulation of adult aging and starvation regulation of postembryonic development.

Chapter 2 addresses the regulation of *pha-4/FoxA* by dietary restriction in the adult. Dietary restriction prolongs the lifespan of adult worms by decreased nutrient signaling through the TOR and Insulin pathways. I found that *pha-4/FoxA* is required for extension of adult lifespan by loss of CeTOR signaling through one of the predicted CeTOR targets, *rsks-1/S6K*.

Chapter 3 addresses the requirement of *pha-4/FoxA* in the response to starvation in the first larval stage. Diverse signaling pathways differentially regulate developmental arrests in response to starvation in different stages. I showed that levels of PHA-4 are important for starvation survival; loss of *pha-4/FoxA* decreased survival and over expression of *pha-4/FoxA* increased starvation survival. *pha-4/FoxA* is required for initiation of development after periods of starvation and is not required for developmental arrest during starvation.

Chapter 4 is a summary of my research and discussion of how my research has provided new ideas about the regulation of FoxA factors by nutrients as well as the transcriptional responses that control both development and aging in response to low nutrients.

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CHAPTER 2

THE TARGET OF RAPAMYCIN PATHWAY ANTAGONIZES PHA-4/FOXA TO CONTROL DEVELOPMENT AND AGING

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Article

The Target of Rapamycin Pathway Antagonizes *pha-4/FoxA* to Control Development and Aging

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Summary

Background: FoxA factors are critical regulators of embryonic development and postembryonic life, but little is know about the upstream pathways that modulate their activity [1]. *C. elegans pha-4* encodes a FoxA transcription factor that is required to establish the foregut in embryos and to control growth and longevity after birth [2–5]. We previously identified the AAA+ ATPase homolog *ruvb-1* as a potent suppressor of *pha-4* mutations [6].
Results: Here we show that *ruvb-1* is a component of the Target of Rapamycin (TOR) pathway in *C. elegans* (CeTOR). Both *ruvb-1* and *let-363/TOR* control nucleolar size and promote localization of box C/D snoRNPs to nucleoli, suggesting a role in rRNA maturation. Inactivation of *let-363/TOR* or *ruvb-1* suppresses the lethality associated with reduced *pha-4* activity. The CeTOR pathway controls protein homeostasis and also contributes to adult longevity [7, 8]. We find that *pha-4* is required to extend adult lifespan in response to reduced CeTOR signaling. Mutations in the predicted CeTOR target *rsks-1/S6 kinase* or in *ife-2/eIF4E* also reduce protein biosynthesis and extend lifespan [9–11], but only *rsks-1* mutations require *pha-4* for adult longevity. In addition, *rsks-1*, but not *ife-2*, can suppress the larval lethality associated with *pha-4* loss-of-function mutations.
Conclusions: The data suggest that *pha-4* and the CeTOR pathway antagonize one another to regulate postembryonic development and adult longevity. We suggest a model in which nutrients promote TOR and S6 kinase signaling, which represses *pha-4/FoxA*, leading to a shorter lifespan. A similar regulatory hierarchy may function in other animals to modulate metabolism, longevity, or disease.

Introduction

Members of the FoxA family of transcription factors encode critical regulators of development, growth, and metabolism. In embryos, FoxA proteins establish the digestive tract and notochord [1, 12] and they contribute to brain development [13, 14]. Postembryonically, FoxA factors control metabolism, developmental progression, and lifespan in response to dietary restriction induced in liquid media or by mutation (*eat-2*) [1, 3, 4, 15]. These functions depend on the appropriate dosage of FoxA activity, and reduced FoxA is associated with developmental abnormalities and disease. For example, animals with a lower dose of FoxA2 in mice or *pha-4* in worms often arrest at birth [5, 16–18]. FoxA2 heterozygotes lose dopaminergic neurons and develop symptoms that resemble Parkinson's disease [13, 14]. The dosage sensitivity of FoxA factors may reflect the contribution of DNA binding site affinity for FoxA target gene selection [5, 19]. Suboptimal DNA binding sites that associate weakly with FoxA may lose occupancy when FoxA levels are reduced.

The susceptibility of FoxA members to changes in concentration suggests that the level and activity of these proteins is tightly controlled, yet we know little about their upstream regulators. Given the involvement of FoxA proteins in metabolism and growth, it is appealing to consider that nutrient signaling pathways might regulate FoxA. Wolfrum and colleagues suggested that insulin induces FoxA2 nuclear exclusion via Akt phosphorylation [1]. However, others have found that FoxA2 associates with target genes in liver nuclei regardless of the status of insulin signaling [1]. Thus, the relationship between FoxA and the insulin pathway is unclear. A second nutrient-sensing pathway is the TOR pathway, which couples growth factors and nutrients to protein homeostasis [20]. Regulation of protein synthesis depends on substrates involved in translation including the eIF4E binding protein 4E-BP and ribosomal S6 kinase (S6K) [20]. TOR also modulates ribosome biogenesis, autophagy, and transcription [20]. *C. elegans* possesses homologs of TOR complex 1 (TORC1) components, including TOR kinase (*let-363* [21]), Raptor (*daf-15* [15]), and LST8 (http://www.wormbase.org/). Both *let-363/TOR* and *daf-15/Raptor* influence larval growth, protein synthesis, adult aging, and autophagy [8, 9, 11, 15, 22]. *let-363* has been implicated in dietary restriction induced by *eat-2* or *pep-2* mutations [9, 23], making it an attractive candidate to function with FoxA. However, little is known about how CeTOR controls growth and aging, or its involvement, if any, with FoxA.

To identify regulators of FoxA factors, we previously undertook a genetic screen for mutations that could suppress the lethality associated with *pha-4*, which encodes the sole *C. elegans* FoxA protein [6]. This screen identified the AAA+ ATPase homolog *ruvb-1* as a potent suppressor of *pha-4* mutations [6]. Here we show that *ruvb-1* is a component of the CeTOR pathway, which establishes a genetic connection between *pha-4/FoxA* and CeTOR. In larvae, both *ruvb-1* and *let-363/TOR* promote box C/D snoRNP localization to the nucleolus, which is required for robust protein synthesis. In adults, inactivation of CeTOR or *rsks-1/S6 kinase* prolongs lifespan, and this effect requires *pha-4* activity. Another regulator of protein translation, *ife-2/eIF4E*, also modulates lifespan but is *pha-4* independent. The data suggest that CeTOR and *rsks-1* antagonize *pha-4/FoxA* to control postembryonic development and adult longevity. Other animals may rely on an analogous regulatory relationship to control metabolism, longevity, or disease states.

Results

Similarity in Phenotypes between *ruvb-1*, a *pha-4* Suppressor, and CeTOR Mutants

We initiated our study by analysis of the loss-of-function phenotype associated with *ruvb-1*. Heterozygous *ruvb-1/+*

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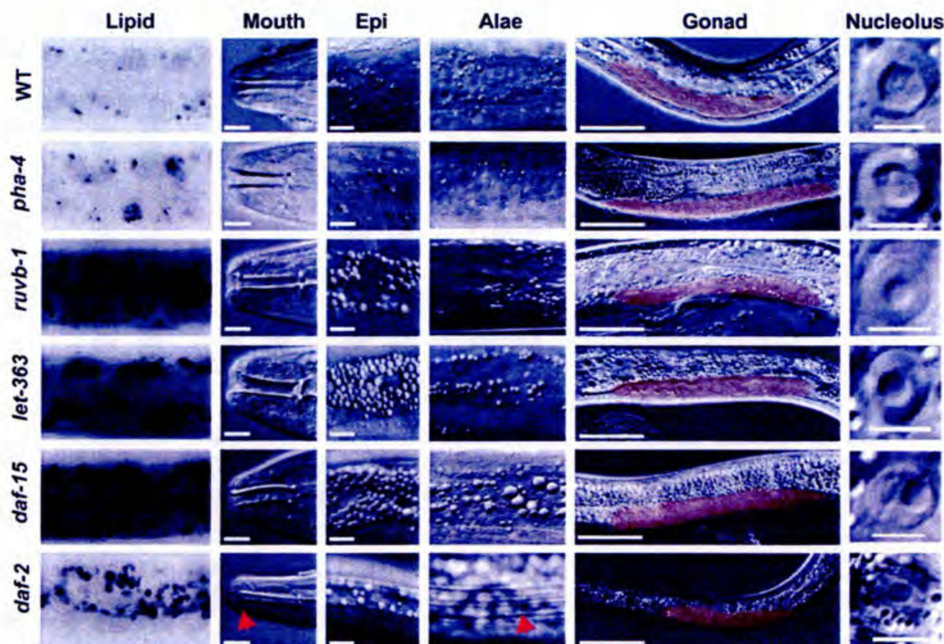

Figure 1. Similarity of Phenotypes Associated with *ruvb-1* and CeTOR

The phenotypes of wild-type (WT), *pha-4(RNAi)*, *ruvb-1(px34)*, *let-363(h111)/TOR*, *daf-15(m81)/Raptor*, and *daf-2(e1370)/insR* mutants were compared at the third larval stage (L3). *daf-2* mutants were examined as Dauer larvae except for lipid accumulation, which was examined in arrested larvae [60]. *pha-4(RNAi)* was initiated at the L1 stage and analyzed at the L3 stage. Lipid accumulation was visualized by Nile Red staining [57]. Mouth opening (arrowhead, scale bars represent 5 μm), epidermal granules (Epi, scale bars represent 5 μm), cuticular alae (arrowhead), arrested gonad development (red, scale bars represent 50 μm), and epidermal nucleoli (scale bars represent 5.12 μm) were examined by light microscopy. Larvae were monitored at 20°C except for *daf-2*, which was temperature sensitive and therefore examined at 25°C.

animals appear wild-type and suppress loss of *pha-4* function [6]. The most striking phenotype associated with *ruvb-1* homozygous mutants is an arrest during the third larval stage (L3), as deduced by body size, perturbed vulval development, and blocked gonadogenesis (Figure 1, Table S1 available online). Previous studies had found that mutations that disrupt insulin signaling (e.g., *daf-2/insR*) and the CeTOR kinase pathway (e.g., *let-363/TOR, daf-15/Raptor)* led to a Dauer or L3 arrest [21, 24], suggesting that *ruvb-1* might belong to one of these pathways. Like *daf-2*, *let-363*, and *daf-15*, *ruvb-1* mutants had increased intestinal lipids and an abundance of granules in the epidermis (Figure 1, Table S1). All four mutants also had small nucleoli, suggesting reduced or defective ribosome biogenesis (Figure 1, Table S1). Further inspection revealed that the *ruvb-1* phenotypes were distinct from those of *daf-2*. For example, *daf-2* mutants have cuticular ridges called alae, the buccal cavity is sealed against the external environment, the intestinal lumen constricts, and the pharynx shrinks radially and ceases to pump [24]. Mutants for *ruvb-1* lacked all of these features: they did not have alae, their digestive tract remained open, and their pharynx continued to pump despite the larval arrest (Figure 1, Table S1). Thus, *ruvb-1* larvae lacked phenotypes typically associated with *daf-2/insR* and resembled *let-363/TOR* mutants.

As a second means to probe the relationship between *ruvb-1*, insulin, and CeTOR, we examined interactions with *daf-16/FoxO*. Mutations in *daf-16* are epistatic to all components of the insulin pathway, but not to those of CeTOR [8, 24]. We found that reduction of *ruvb-1* by RNAi led to an L3 arrest, high fat, and epidermal granules even in combination with a null allele of *daf-16* (Figure 2A). This result indicates that *ruvb-1* functions independently of *daf-16*.

Next we examined the subcellular localization of DAF-16. Normally, insulin signaling promotes cytoplasmic retention of a DAF-16::GFP reporter, whereas reduced insulin signaling leads to nuclear accumulation [25–28]. DAF-16::GFP remained cytoplasmic in *ruvb-1(RNAi)*, *pha-4(RNAi)*, or *let-363(RNAi)*; *daf-15(RNAi)* animals (Figure 2B), suggesting that *ruvb-1* functions in parallel or downstream of the insulin pathway. As a control, we observed nuclear DAF-16::GFP when animals were starved, in agreement with previous studies [25, 28] (Figure 2B). In sum, the constellation of phenotypes and genetic interactions we observed for *ruvb-1* was identical to those associated with *let-363/TOR* and its interacting partner *daf-15/Raptor* [15, 21, 29]; no other known pathway has

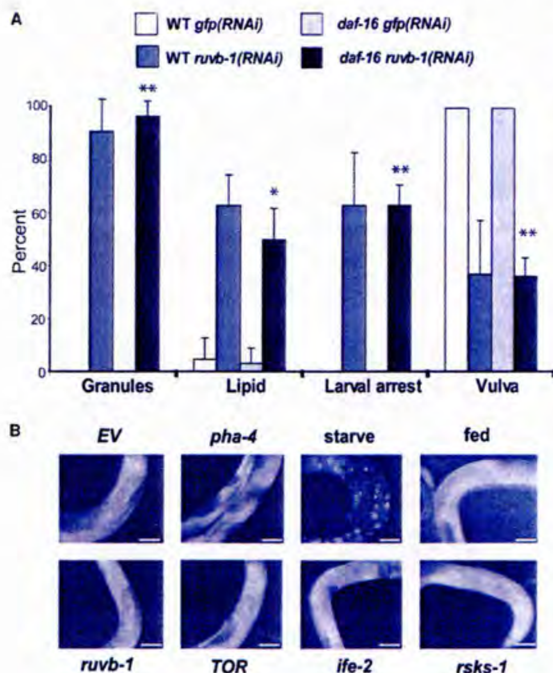

Figure 2. Genetic Interactions between Fox Factors and *ruvb-1* or CeTOR

(A) *ruvb-1* phenotypes do not depend on *daf-16/FoxO*. RNAi against *GFP* or *ruvb-1* was induced in either wild-type (WT) or *daf-16(mu86)* hermaphrodites, and their progeny scored by light microscopy for larval arrest, epidermal granules (Granules), or a mature vulva. Lipid accumulation of L3 progeny was determined by Nile Red staining [57] (20°C, 3 experiments, $n \geq 24$ animals for each condition, error bars denote standard deviation, $^{**}p < 0.0002$, $^{*}p = 0.0038$).

(B) DAF-16 localization was monitored with *daf-16p::daf-16::GFP* [25]. Worms subjected to OP50 (fed), empty vector (EV), *ruvb-1(RNAi)*, *pha-4(RNAi)*, *ife-2(RNAi)*, *rsks-1(RNAi)*, or *let-363(RNAi); daf-15(RNAi) (TOR)* show cytoplasmic localization compared to worms starved for 24 hr, which display nuclear localization ($n \geq 9$ worms for each condition, scale bars represent 50 μm).

a matching set of attributes in *C. elegans*. We conclude that *ruvb-1* is a likely component of the CeTOR pathway.

ruvb-1 and CeTOR Are Critical for Box C/D snoRNP Localization

How might *ruvb-1* contribute to the CeTOR pathway? Biochemical studies in other organisms have identified RUVB orthologs as members of several multiprotein complexes [30–32]. To assess which of these complexes could account for the role of *ruvb-1* in the CeTOR pathway, we screened members of these complexes by RNAi to determine whether any were associated with *let-363/TOR*-like phenotypes (Table S2, Figure S3). Inactivation of *nol-5*, K07C5.4, or *fib-1* each resulted in a larval arrest with excess epidermal granules and small nucleoli (Table S2). We did not observe a high-fat phenotype for *nol-5*, K07C5.4, or *fib-1*, indicating that either a separate RUVB-1-containing complex is responsible for lipid accumulation or that inactivation of these four genes affects a common target to different extents. The similarity of phenotypes suggested that *nol-5*, K07C5.4, and *fib-1* could account for at least part of the CeTOR phenotype of *ruvb-1*.

nol-5, K07C5.4, and *fib-1* encode proteins predicted to be members of the box C/D snoRNP [33]. Box C/D snoRNPs function in the nucleolus and methylate pre-rRNAs during ribosome maturation [33]. In other organisms, the box C/D snoRNP complex is assembled and stabilized in the nucleus by association with multiple proteins, including RUVB [31, 34]. Once stabilized, the mature box C/D snoRNP is transported into the nucleolus where it methylates rRNAs [32]. We used an antibody to the predicted box C/D snoRNP component FIB-1 to localize box C/D snoRNPs in wild-type and mutant worms. We observed robust FIB-1 in 100% of the nucleoli of wild-type animals, and this signal was lost after *fib-1(RNAi)*, indicating that the stain was specific (Figure 3). FIB-1 levels were reduced, and FIB-1 failed to localize to the nucleolus in the majority of *ruvb-1* mutant larvae (Figure 3). In affected animals, we observed a faint ring of FIB-1 at the nucleolar periphery and, to a lesser degree, within the nucleoplasm. Thus, *C. elegans ruvb-1* is required for snoRNP localization and accumulation, similar to its human and yeast counterparts. Inactivation of *let-363/TOR* produced similar defects, with low levels of FIB-1, which were localized to the nucleoplasm and nucleolar periphery (Figure 3). This result is consistent with *ruvb-1* and CeTOR functioning in the same pathway. We also tested whether snoRNP localization required *pha-4* activity. FIB-1 localization was unperturbed by *pha-4(RNAi)* (Figure 3). Moreover, *pha-4(RNAi)* did not restore FIB-1 localization to *ruvb-1* or *let-363* mutants (Figure S1). The data indicate that PHA-4 is not involved in box C/D snoRNP localization.

TOR is responsive to nutrient status [20], and CeTOR may be part of the dietary restriction (DR) pathway for lifespan extension [9, 23], suggesting that FIB-1 localization might be regulated by nutritional status. We analyzed worms that had undergone 8 hr starvation and observed that FIB-1 levels were reduced and restricted to the nucleoplasm (Figure 3). Regulation of FIB-1 was specific, as shown by the fact that nucleolar FIB-1 was observed in *daf-2/insR*, *rsks-1/S6 kinase*, and *ife-2/eIF4E* mutants (Figure 3). These data reveal that food and CeTOR signaling promote accumulation of FIB-1 in the nucleolus and by extension rRNA maturation.

Given the effect of *let-363/TOR* and *ruvb-1* on snoRNP localization, we predicted that protein biosynthesis should require these factors, as well as box C/D snoRNP components. *fib-1* or TOR pathway members were inactivated in adult worms, and ^{35}S incorporation monitored over a 5 hr period. Reduction of *let-363* led to a 50% decrease in ^{35}S incorporation, in agreement with previous studies (Figure 4) [9, 11]. We observed a 20% decrease in ^{35}S incorporation when we inactivated *pha-4*, genes required for the box C/D snoRNP (*ruvb-1* or *fib-1*)

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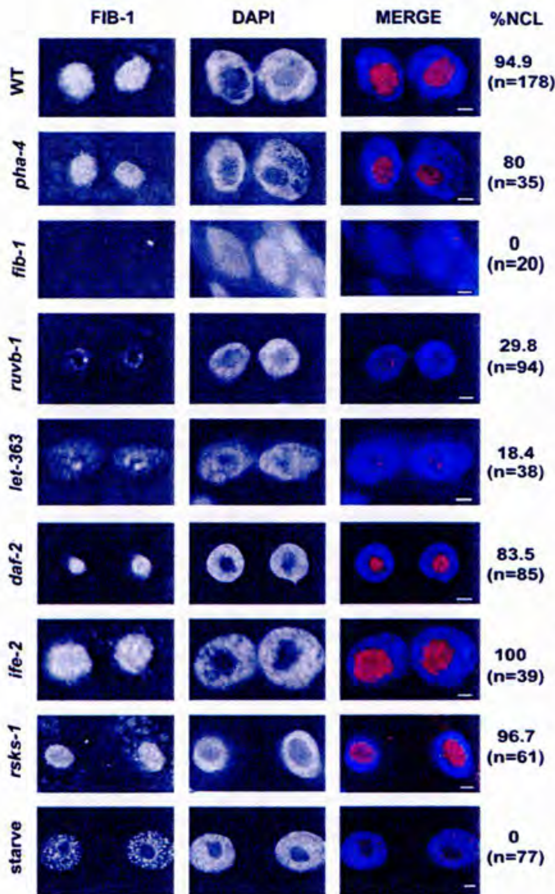

Figure 3. Localization of the Box C/D snoRNP Complex Requires Food, *ruvb-1*, and *let-363/TOR*

Fed (WT) or starved (8 hr, starve) wild-type, *pha-4(RNAi)*, *ruvb-1(px34)*, *let-363(h111)/TOR*, *daf-2(e1368ts)/InsR*, *rsks-1(ok1255)*, or *ife-2(ok306)/eIF4E* larvae were stained for FIB-1 (pink) and DNA (DAPI, blue) at the L3 stage (scale bars represent 2 μm). *pha-4(RNAi)* was initiated at the L1 stage and analyzed at the L3 stage. *fib-1(RNAi)* was initiated at the L4 stage and L1 progeny were analyzed. Data were quantified for percent nucleolar (% NCL) and number of nuclei (n).

or *ife-2* for translation initiation (Figure 4). An empty vector controlled that produced a small, nonspecific double-stranded RNA (dsRNA) served as a negative control (Figure 4). These data suggest that one way CeTOR controls protein biosynthesis is by modulating the accumulation and localization of box C/D snoRNPs. In addition, *pha-4* is required for protein synthesis independent of the box C/D snoRNP complex.

let-363/TOR and *daf-15/Raptor* Antagonize *pha-4*

ruvb-1 was originally discovered as a suppressor of the lethality associated with partial inactivation of *pha-4* [6], prompting us to test whether *let-363/TOR* and *daf-15/Raptor* could also suppress *pha-4*. We examined suppression in two ways. First, we used RNAi to inactivate *let-363/TOR* and *daf-15/Raptor*, and we examined the effect of reduced CeTOR signaling on *pha-4* mutants. We engineered *pha-4* to be cold sensitive (*pha-4(ts)*; Experimental Procedures) and chose an intermediate temperature when worms die because of intermediate levels of PHA-4 protein (20°C; [16]). The intermediate temperature provided a sensitive means to uncover genetic interactions between CeTOR pathway components and *pha-4*. We inactivated *let-363* and *daf-15* together, to ensure the strongest possible reduction in CeTOR signaling, and scored the proportion of *pha-4(ts)* progeny that progressed past the first larval stage (L1). We found that the fraction of survivors for *pha-4(ts); let-363(RNAi); daf-15(RNAi)* was similar to that observed for *pha-4(ts); ruvb-1(RNAi)* and about 2-fold higher than *pha-4(ts)* alone (Figure S2). This result suggests that inactivation of canonical CeTOR pathway components suppresses *pha-4* mutations.

Second, we examined survival of *let-363/+* or *daf-15/+* heterozygotes treated with *pha-4* dsRNA. To sensitize our ability to detect genetic interactions, *pha-4* dsRNA was diluted with GFP dsRNA to generate a partial inactivation of *pha-4* (Experimental Procedures). Alone, *let-363/+* and *daf-15/+* animals appeared superficially wild-type, which allowed us to score the number of animals that lived when subjected to *pha-4(RNAi)* [6] (data not shown). This experiment revealed that approximately twice as many *let-363/+; pha-4(RNAi)* larvae lived compared to *pha-4(RNAi)* alone (Figure S2). *daf-15/+* heterozygotes failed to rescue *pha-4(RNAi)* to a significant extent, which may reflect distinct roles for *ruvb-1*, *let-363*, and *daf-15*, or dissimilar genetics such as maternal effects or genetic dominance [6]. Together, the data show that reduced CeTOR activity (i.e., *let-363/TOR* or *ruvb-1*) can suppress the lethality associated with reduced *pha-4* and that therefore the CeTOR pathway antagonizes *pha-4* during development.

pha-4 Is Required for Lifespan Extension in Response to Decreased CeTOR

Reduced CeTOR signaling leads to prolonged lifespan whereas reduced *pha-4* shortens life, and both genes are implicated in dietary restriction [3, 8, 9, 11, 15]. To explore the relationship between these genes, we inactivated both *pha-4* and CeTOR components conditionally, beginning at the fourth larval stage and continuing through adulthood. Alone, *pha-4(ts)* animals had a slightly shortened lifespan, as had been observed previously [3]. Reduction of *let-363* and *daf-15* together led to a statistically significant increase in lifespan in 3/4 experiments ($p < 0.05$; Figure 5A, Table S4). To examine the effect of *pha-4*, we analyzed our data sets by multivariate Cox

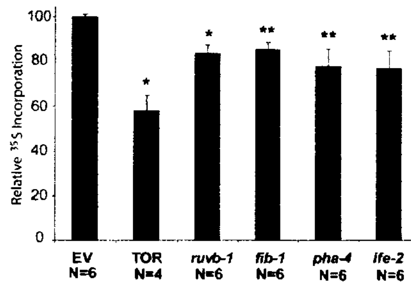

Figure 4. Inhibition of CeTOR or Box C/D snoRNPs Decreases the Rate of Newly Synthesized Proteins

Relative levels of ^{35}S-methionine incorporation in 2-day-old *fog-1(q253ts)* adult animals treated with either empty vector (EV), *let-363(RNAi); daf-15(RNAi) (TOR)*, *ruvb-1(RNAi)*, *fib-1(RNAi)*, *pha-4(RNAi)*, or *ife-2(RNAi)*. RNAi was initiated at the L4 stage (day 0 of adulthood) at 25 C. Bar graphs represent average ^{35}S-methionine incorporation normalized to total protein levels for different RNAi treatments compared to EV(RNAi) (**$p < 0.006$, *$p < 0.0002$, one-sided paired t test. Error bars represent SEM; N, number of measurements).

regression modeling, which allowed four experimental conditions to be compared simultaneously [35]. This analysis revealed that *pha-4* was required for CeTOR-induced lifespan extension because we observed a statistically significant decrease in longevity for *pha-4* with reduced CeTOR compared to *pha-4* alone ($p < 0.0001$; Figure 5A, Table S4, Figure S5). Strikingly, *pha-4(ts); let-363(RNAi); daf-15(RNAi)* animals had shorter life spans than *pha-4(ts)* alone. This result suggests that *pha-4* is crucial for survival when the TOR pathway is inactivated. The effect of *pha-4* on longevity was specific because *pha-4* was not required for the pronounced lifespan extension induced by *daf-2/insR* mutations (Figure 5B, Table S4), in agreement with previous studies [3]. Moreover, inactivation of *pha-4* had only minor effects on the number of eggs laid, indicating that these worms were generally healthy and fecund (115 ± 37 eggs for *pha-4(RNAi)* mothers versus 127 ± 33 for wild-type [n = 18]). We conclude that *pha-4/FoxA* is selectively required for lifespan extension by reduced CeTOR.

To investigate how CeTOR signaling negatively regulates *pha-4*, we examined whether PHA-4 levels or localization changed in response to CeTOR inactivation, starvation, or aging. By monitoring a translational PHA-4::mCherry reporter in adults, we observed strong expression in the pharynx and lower levels in the intestine under all experimental conditions (Figure S4). We also observed constitutively nuclear expression in agreement with Panowski et al. and Zhang et al., but distinct from Wolfrum et al. [1, 3]. Our data suggest that the levels of nuclear PHA-4 protein do not change in response to aging or starvation. Instead, the transcriptional activity may be altered by CeTOR signaling.

rsks-1 Antagonizes *pha-4*

The TOR pathway controls protein homeostasis at many levels, including ribosome biogenesis, translation, and autophagy [20]. We wondered which of these downstream pathways relied on *pha-4* for lifespan extension. One appealing candidate was *rsks-1*, which is homologous to the TOR target S6 kinase [20] and which leads to lifespan extension when inactivated in *C. elegans* [9–11]. We observed a ~10% extension in lifespan for *rsks-1(ok1255)* in 4/4 experiments, similar to previous studies (Figure 5C, Table S5) [10]. This effect was dependent on *pha-4* as shown by the fact that *pha-4(RNAi)* caused worse survival in *rsks-1* compared to wild-type ($p = 0.007$ by multivariate Cox modeling [35]; Figure 5C, Table S5, Figure S5).

Next we tested *ife-2*, which encodes one of five eIF4E isoforms (WS180; http://www.wormbase.org). In other animals, TOR promotes eIF4E activity by inactivating the eIF4E repressor 4E-BP [20]. In 3/3 experiments, we observed extended longevity for *ife-2* mutants (Figure 5D, Table S5), similar to previous work [9, 11]. Lifespan decreased when *pha-4* was inactivated by RNAi (4/5 experiments; Figure 5D, Table S5). However, inactivation of *pha-4* reduced longevity to a similar extent as inactivation of *pha-4* in wild-type animals and never returned *ife-2* lifespan to baseline ($p = 0.158$ by multivariate Cox modeling [35]; Table S5, Figure S5). These data implicate alternative processes for lifespan extension by *ife-2*. We suggest that *pha-4/FoxA* plays a critical role for lifespan extension because of decreased *let-363* and *rsks-1*, but not *ife-2*.

We were surprised that *rsks-1* and *ife-2* had different genetic interactions with *pha-4* for adult aging. To extend this finding, we tested whether *rsks-1* or *ife-2* mutations could suppress the larval lethality associated with loss of *pha-4* function. *pha-4* was partially inactivated by RNAi in wild-type, *rsks-1*, or *ife-2* mutants. We observed a ≥2× suppression of *pha-4* by *rsks-1* but no suppression by *ife-2* (Figure 5E, Table S6). These results bolster the conclusion that *rsks-1* is a negative regulator of *pha-4*, whereas *ife-2* is not.

Discussion

We have identified the *pha-4* suppressor *ruvb-1* as a new component of the *C. elegans* TOR pathway, which led us to probe the genetic interactions between CeTOR and *pha-4*. Our findings reveal that CeTOR antagonizes *pha-4* during larval development and adult aging. *let-363/TOR* and *ruvb-1* are both needed to accumulate box C/D snoRNPs in the nucleolus, and loss of these proteins leads to decreased protein synthesis. Downstream of CeTOR, reduced *rsks-1/S6* kinase, but not *ife-2/eIF4E*, relies on *pha-4* activity to prolong life. Moreover, *rsks-1/S6* kinase mutations, but not *ife-2/eIF4E*, can suppress *pha-4*-associated larval lethality. These data suggest that nutrients activate the CeTOR pathway and *rsks-1/S6* kinase to repress *pha-4* during larval development and adult aging (Figure 6). Reduced nutrients, for example during dietary restriction, leads to enhanced *pha-4/FoxA* activity and prolonged lifespan, via reduced TOR signaling.

CeTOR and *ruvb-1* Share Common Phenotypes

We have shown that *ruvb-1* and *let-363/TOR* control the accumulation and localization of box C/D snoRNPs within nucleoli, providing an explanation for *ruvb-1* function in the CeTOR pathway. Box C/D snoRNPs methylate rRNAs during maturation, and loss of box C/D snoRNPs is predicted to reduce ribosome biogenesis. This function likely explains, at least in part, why inactivation of CeTOR [9, 15, 21] or *ruvb-1* (this study) leads to decreased protein biosynthesis and arrested larval development. In our hands, CeTOR had the most pronounced effect on protein synthesis rates compared to other genes, which may reflect multiple levels of regulation of protein homeostasis by CeTOR, similar to other organisms [20]. By contrast, *rsks-1/S6 kinase* and *ife-2/eIF4E* each promoted protein biosynthesis, but neither was necessary to complete larval

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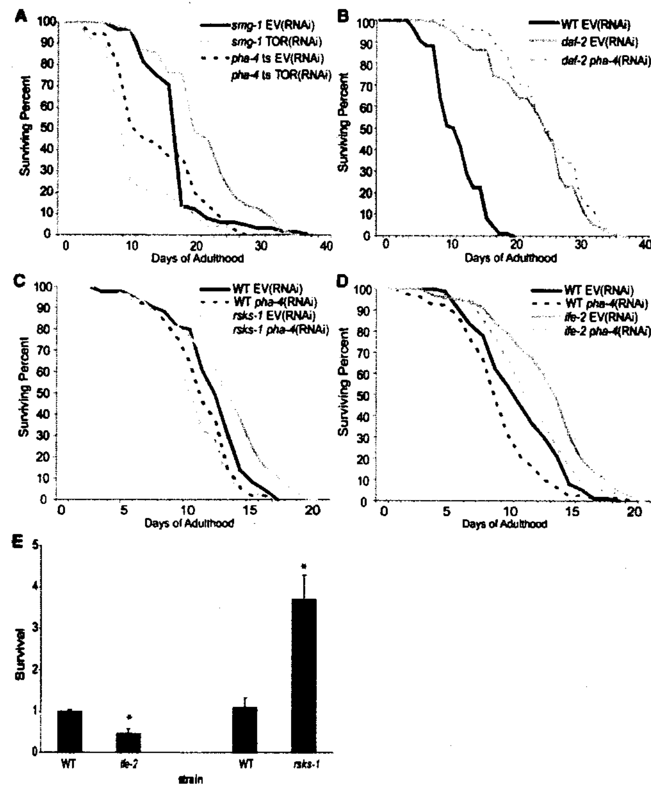

Figure 5. Longevity Resulting from Reduced CeTOR or *rsks-1* Requires *pha-4*

(A–D) Survival curves.

(A) *pha-4(zu225);smg-1(cc546ts)* (*pha-4(ts)*) or *smg-1(cc546ts)* grown at 24°C and shifted to 15°C beginning at the L4 stage. Worms were subjected to *let-363(RNAi)*; *daf-15(RNAi)* (*TOR(RNAi)*) to inactivate TOR signaling compared to empty vector control (EV). Mean lifespan was 18.1 days for *smg-1; EV(RNAi)* (control), 21.3 days for *smg-1; TOR(RNAi)* (p = 0.0077 versus control), 13.8 days for *pha-4(ts); EV(RNAi)* (p = 0.0017 versus control), and 12.2 days for *pha-4(ts); TOR RNAi* (p = < 0.0001 versus control).

(B) *daf-2(e1368ts)* worms were grown at 15°C and shifted to 25°C beginning at the L4 stage to inactivate insulin signaling. Worms were subjected to *pha-4(RNAi)* or an empty vector control (EV). Wild-type (WT) worms were grown at 25°C. Mean lifespan was 11.8 days for *WT; EV(RNAi)* (control), 23.6 days for *daf-2; EV(RNAi)* (p < 0.0001 versus control), and 25.2 days for *daf-2; pha-4(RNAi)* (p < 0.0001 versus control).

(C) Wild-type (WT) and *rsks-1(ok1255)* worms were grown at 25°C. Worms were subjected to *pha-4(RNAi)* or an empty vector control (EV). Mean lifespan was 11.96 days for WT; *EV(RNAi)* (control), 11.1 days for WT; *pha-4(RNAi)* (p = 0.0075 versus control), 12.98 days for *rsks-1; EV(RNAi)* (p = 0.0011 versus control), 10.4 days for *rsks-1; pha-4(RNAi)* (p = 0.0001 versus control).

(D) Wild-type (WT) and *ife-2(ok306)* worms were grown at 25°C. Worms were subjected to *pha-4(RNAi)* or an empty vector control (EV). Mean lifespan was 11.16 days for WT; EV(RNAi) (control), 9.5 days for WT; *pha-4(RNAi)* (p = 0.0032 versus control), 13.6 days for *ife-2; EV(RNAi)* (p = < 0.0001 versus control), 12.15 days for *ife-2; pha-4(RNAi)* (p = 0.0664 versus control). In all experiments, RNAi was initiated at the L4 stage [61].

(E) Suppression of the lethality associated with reduced *pha-4* by *rsks-1, not ife-2*. Wild-type animals and animals mutant for *ife-2(ok306)* or *rsks-1(ok1255)* were subjected to weak *pha-4(RNAi)* (Experimental Procedures). The proportion of mutant animals that survived beyond the L1 stage was counted and normalized against wild-type worms also subjected to *pha-4(RNAi)* (25°C, 2 experiments, n ≥ 800 animals for each strain, error bars denote standard error, *p = 0.001).

development or to localize FIB-1 ([9–11], this study). *C. elegans* possesses five predicted eIF4E factors [36], which may explain why *ife-2/eIF4E* was associated with weaker phenotypes than *ruvb-1* or CeTOR.

CeTOR and one of the core components of the box C/D snoRNP complex, W01B11.3/Nop58, have been implicated in lifespan extension [8, 37], raising the question of whether *ruvb-1* or other snoRNP components contribute to longevity as well. Inactivation of *ruvb-1* beginning at the L4 stage did not cause a reproducible extension of lifespan (Table S3). One possibility is that *ruvb-1* and the box C/D snoRNP may be important for lifespan extension, but pleiotropic

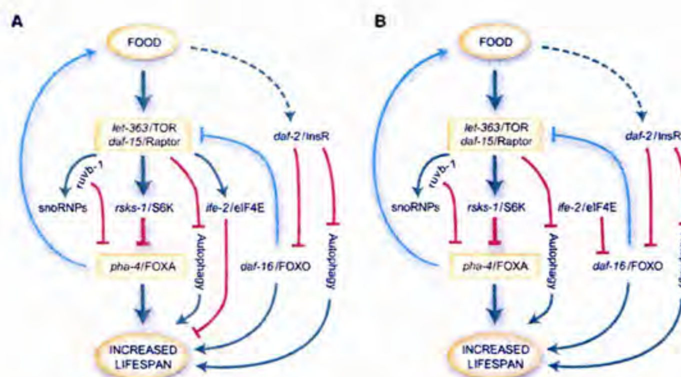

Figure 6. Two Models for Lifespan Extension Resulting from Reduced Protein Synthesis in Adults
Genetic epistasis suggests that *pha-4/FoxA* acts downstream of *rsks-1/S6 kinase* and *let-363/TOR*. *ife-2/eIF4E* may act downstream of TOR (A) or parallel to TOR (B). Green arrows depict positive regulation, red lines denote negative regulation, and blue lines depict feedback. See the Discussion for details.

phenotypes associated with other RUVB-1-containing complexes may mask a role in aging. Different experimental conditions may reveal an aging role.

Models for Lifespan Extension by Reduced Protein Biosynthesis

Large-scale screens in *C. elegans* have identified a multitude of genes that can modulate adult lifespan (reviewed in [38]). An ongoing challenge is to organize these genes into defined pathways. Analysis has been complicated by the large number of genes that can impinge on aging, the complex genetic interactions among these genes, the different approaches to induce lifespan extension by DR, and the need for conditional or partial inactivation of essential genes [3, 39–44]. Previous studies have shown that reduction of CeTOR or the translation machinery can extend lifespan [8–11, 45, 46]. Given that CeTOR controls protein homeostasis and responds to nutrients [9, 20, 23], one might predict that inhibition of translation would extend longevity by the same pathway as reduction of CeTOR signaling. However, genetic studies have led to seemingly contradictory conclusions regarding the relationship between TOR and protein translation, or between these factors and DR [9–11, 25, 45]. *pha-4* epistasis offers another approach to group genes within common pathways. Based on our analysis with *pha-4/FoxA* and results from other studies, we suggest two models for coupling nutrients to protein translation and aging (Figure 6). These models both place *pha-4* downstream of S6 kinase and CeTOR, but differ with regard to positioning *ife-2* and the translation initiation factors within an aging network. We recognize that the two models are not mutually exclusive and that the translation initiation factors may function in multiple contexts.

For both models, we place CeTOR and *rsks-1/S6 kinase* downstream of nutrients, based on genetic epistasis experiments in *C. elegans* [9, 10, 23], common effects on snoRNP localization and protein biosynthesis (this study, [9, 10]), and studies with other species [20]. Inactivation of *rsks-1* and CeTOR together resembles inactivation of CeTOR alone for lifespan extension [9], consistent with these genes functioning in a common pathway. Moreover, neither gene is dependent on *daf-16/FoxO* [9, 10], suggesting that this pathway is parallel or downstream of insulin signaling. The genetic interactions between *pha-4/FoxA* and either CeTOR or *rsks-1* tentatively place *pha-4* downstream of these kinases. We position *ruvb-1* and snoRNPs downstream of CeTOR but separate from *rsks-1* or *ife-2*, based on a lack of aging phenotypes for *ruvb-1* and common snoRNP phenotypes for *ruvb-1* and *let-363*, but not *ife-2* or *rsks-1*. Finally, we draw a dotted arrow between food and the insulin pathway, to reflect examples of *daf-16* regulation or function during starvation or dietary restriction induced by some means but not others [25, 28, 40].

In the first model, we position *ife-2/eIF4E* and presumably additional translation initiation factors downstream of CeTOR (Figure 6A). In other organisms, 4E-BP is a negative regulator of translation initiation that binds and sequesters eIF4E [20]. TOR phosphorylates 4E-BP, leading to eIF4E release. If a similar regulatory hierarchy exists in worms, CeTOR may activate translation initiation machinery. An advantage of this model is that all genes that affect protein biosynthesis (CeTOR, *rsks-1*, translation initiation factors, *pha-4*, as well as food deprivation; this study, [9–11]) are consigned to one branch of the aging network.

A critical feature of the first model is a pair of feedback loops. In the first loop, *pha-4* promotes food uptake (Figure 6A, blue; [2]), which may explain why inactivation of *pha-4* leads to reduced protein synthesis. In the second feedback loop, *daf-16* negatively regulates *daf-15/Raptor* [15] to modify CeTOR activity (Figure 6A, blue). This feedback loop may explain two perplexing genetic interactions. First, there have been differing claims regarding the dependence on *daf-16/FoxO* for lifespan extension after inactivation of translation initiation factors [9–11, 37, 45]. We suggest that inactivation of *daf-16* may increase CeTOR activity, which is predicted to boost translation and thereby suppress *ife-2/eIF4E* mutations. This scenario can explain genetic interactions between *daf-16* and *ife-2* (or other translation factors) [9, 11], despite the absence of nuclear-enriched DAF-16 (Figure 2). Second, although DR and CeTOR appear largely independent of insulin

signaling for aging [8, 41, 43], *let-363/TOR* and *daf-2/InsR* fail to synergize for lifespan extension when they are inactivated together [8, 9]. One possibility is that the negative feedback loop decreases CeTOR activity in *daf-2* mutants, such that *daf-2* single mutants resemble *daf-2; let-363* double mutants for aging (Figure 6A, blue). We note, however, that *daf-2* mutants do not alter protein synthesis rates [9] or suppress *pha-4* mutations (data not shown), suggesting that *daf-16* does not repress TOR completely or in all cells.

A second model separates *ife-2* and the translation-initiation factors from CeTOR, where they impinge on *daf-16* more directly (Figure 6B). For example, the absence of food or inactivation of translation factors may induce a stress response that activates DAF-16. In yeast, one of the two isoforms of eIF4E is upregulated by stress and required for the stress response [47]. *C. elegans* also possesses multiple isoforms of eIF4E [36], and future studies will determine whether any of these isoforms are involved in stress. A link to stress may explain why inactivation of some translation factors impacts the nuclear localization of DAF-16 to some extent [45]. A stress response may also explain why food deprivation induces nuclear localization of DAF-16 [25, 28], even though dietary restriction does not [40].

Both DR and TOR are important regulators of autophagy, which cells use to survive periods of starvation by recycling macromolecules and nutrient transporters [48]. Autophagy is necessary for lifespan extension resulting from mutations that inactivate feeding (*eat-2*), CeTOR, or insulin signaling throughout the life of the animal [22, 49–52]. However, inactivation of *let-363/TOR* in adults, rather than throughout life, can prolong lifespan in the absence of an obvious autophagic response [22]. Neither *ife-2/eIF4E* nor *rsks-1/S6K* mutants have increased autophagy, yet they extend longevity [22]. Induction of autophagy in *daf-2/InsR; daf-16/FoxO* mutants is not sufficient for increased lifespan [22]. These three observations suggest that there must be additional processes beyond autophagy involved in lifespan extension and that these processes depend on *pha-4/FoxA*. *pha-4* can also impinge on autophagy. Long-term reduction (>1 generation) of *pha-4* blocks the induction of autophagy in response to long-term reduction in feeding *(eat-2)* or *daf-15/Raptor* heterozygotes [22]. These effects could reflect a developmental role for these proteins, particularly *pha-4*, which is required for embryonic and larval development [4, 5]. Alternatively, *pha-4* may be an acute regulator of the autophagic response. Comparison of long-term versus short-term inactivation of these proteins will clarify their roles.

In summary, our analysis of *ruvb-1* has revealed that the activity of *pha-4* is modulated by the CeTOR pathway. This interaction could be relatively direct, for example, by PHA-4 modification. Alternatively, it could be indirect, if both *pha-4* and CeTOR impinge on common processes. An intriguing avenue for future studies will be to determine whether the developmental or metabolic roles of FoxA proteins in other animals are modified by TOR signaling.

Experimental Procedures

See Supplemental Data for additional experimental procedures.

RNA Interference

RNAi by bacterial feeding was performed essentially as described in [6]. HT115 bacteria [53] expressing dsRNA for *GFP*, *ruvb-1*, *pha-4*, *let-363*, *daf-15*, *dpy-1*, *nhr-23*, *fib-1*, *rsks-1*, or *ife-2* were grown in overnight cultures and seeded onto plates containing 5 mM IPTG (Sigma) and 60 μg/ml Carbenicillin (Sigma) or 1 mM IPTG only for lifespan analysis. All RNAi clones were derived from the Ahringer library [54] except for *pha-4* (bSEM 865) [55], *GFP* [53], *let-363* (bSEM 911), *daf-15* (bSEM 912), and *rsks-1*, obtained from the *C. elegans* ORF-RNAi library v1.1 (Geneservice Ltd.). Clones were confirmed by restriction enzyme digest.

daf-16 Epistasis

Wild-type or *daf-16(mu86)* [56] hermaphrodites were subjected to *ruvb-1(RNAi)* beginning at the L4 stage. Progeny were scored for phenotypes after 3–4 days incubation at 20°C. L3 larval arrest was determined by body size, gonad extension, and presence/absence of a mature vulva with either wild-type or protruding morphology. Fat was detected by Nile Red staining [57] and scored if increased from average wild-type staining by visual inspection. Epidermal granules are refractile storage vesicles in the epidermis and were scored for increased abundance compared to the wild-type.

Immunostaining

Immunostaining was performed as described previously [58] with the following changes. Microscope slides were treated with a 0.1% poly-L-lysine solution overnight (Sigma-Aldrich Product #P8920). In situ antibody staining for FIB-1 was performed with a 1:200 dilution of α-FIB-1 mouse monoclonal antibody (EnCor BioTechnology, Catalog #MCA-38F3) and detected with a 1:200 Cy3 conjugated α-IgG secondary antibody (Jackson ImmunoResearch). Mounting medium consisted of 50% glycerol in PBS with DAPI and p-phenylenediamine.

Worms were subjected to *pha-4(RNAi)*, *Empty Vector(RNAi)*, or OP50 bacteria at the L4 or L1 stage. L1 progeny or L3-stage worms were picked off of plates, washed with water, and placed in 2% paraformaldehyde and only L3 worms were cut to release gonad and intestine. Worms were fixed in 2% paraformaldehyde and permeabilized by freeze-crack method for 30 min, then submersed in ice-cold methanol for 3 min. After methanol treatment, slides were rinsed twice, 5 min each, in 1X TBST (Tris-Buffered Saline Tween). Slides were later blocked for 30 min in TNB (0.1 M Tris HCl, 0.15 M NaCl, 0.05% Tween 20 [pH 7.5] containing blocking reagent [NEN]) and 10% NGS (Normal Goat Serum) followed by overnight incubation with the primary antibody at 15°C. After overnight incubation, slides were washed 3 times in 1X TBST and secondary antibodies were added. Slides were incubated with the secondary antibody at room temperature for 2 hr. Images were captured with DeltaVision RT Deconvolution system and SoftWoRx software (Applied Precision).

Lifespan Analysis

Lifespan analysis was performed as described previously [9] with the following changes. Worms were grown for at least 2 generations at 25°C or 20°C, as indicated, before the experiment was initiated. Hermaphrodites were allowed to lay eggs for 4–8 hr on OP50, and progeny were grown to the L4 stage. In all experiments, L4 larvae were transferred to new plates with 1 mM IPTG and appropriate bacteria to initiate *pha-4(RNAi)* or *ruvb-1(RNAi)* versus a vector control. The first day of adulthood was counted as day one of the experiment. Worms were moved daily until reproduction ceased. Worms were moved every 3–4 days for the rest of the lifespan assay. Lifespan analysis was conducted with wild-type, *daf-2(e1368)* [59], *ife-2(ok306)* [36], and *rsks-1(ok1255)* [10].

For experiments with reduced CeTOR, *smg-1(cc546ts); pha-4(zu225)* [16] and control *smg-1(cc546ts)* (http://www.addgene.org/labs/Fire/Andrew/Vec97.pdf) worms were used. Worms were grown at the permissive temperature of 24°C for at least two generations prior to beginning the experiment. Hermaphrodites were allowed to lay eggs for 4–6 hr and removed. L4 progeny were moved to the nonpermissive temperature of 15°C and RNAi was initiated. We used a 1:1 mixture of bacteria for *let-363(RNAi); daf-15(RNAi)*. The first day of adulthood was counted as day one of the experiment. Worms were moved every other day until reproduction ceased. Worms were moved every 5–7 days for the rest of the lifespan assay. Censoring within each experiment included animals that ruptured, crawled off the plate, or exhibited progeny hatching internally. We have reported the number of ruptured animals.

Statistical Methods for Lifespan Analysis

Log rank tests were used in pair-wise comparisons of wild-type, mutant, RNAi, and control groups. To determine whether *pha-4* had an effect beyond its effect on wild-type worms, we combined data from multiple experiments with the same conditions and applied multivariate Cox proportional modeling (Cox regression) with Stata Software [35]. This statistical approach enabled us to compare four experimental conditions at once and

determine whether *pha-4* had a greater effect on our experimental strain relative to the wild-type control. It also included information from the censored subjects.

Suppression of *pha-4*

pha-4(RNAi) suppression was performed as reported previously [6] with bacteria expressing *pha-4* and *GFP* dsRNA at a ratio of 1:4 or 1:8, to give an intermediate inactivation of *pha-4*. 5–10 L4 stage wild-type, *unc-42 ruvb-1(px34)/evl-1, let-363(h111)/dpy-5, daf-15(m81)/ unc-24, ife-2(ok306)*, and rsks-1*(ok1255)* worms were picked to 2–4 RNAi plates per experiment and incubated at 25°C. P_0 were allowed to lay eggs for 1 day and then removed. Progeny were counted 2 days later for the percentage of animals older than L1 ($n \geq 100$ animals/plate). p values were determined by t test.

pha-4(ts) suppression was performed as reported previously [6] with bacteria expressing *ruvb-1(RNAi)*, a mix of *let-363(RNAi); daf-15(RNAi)*, or bacteria containing empty vector as a control. Ten L4 *pha-4(ts)* worms were picked to 2–5 RNAi plates per experiment and incubated at 20°C. P_0 were allowed to lay eggs for 1 day and then removed. Progeny were counted 2 days later for the percentage of animals older then L1 ($n \geq 20$ animals/plate). Strength of RNAi was monitored by observation of progeny for larval arrest (*ruvb-1(RNAi))* or slow growth and sterility (*TOR(RNAi))*. p values were determined by t test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, and six tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/18/1355/DC1/.

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CHAPTER 3

PHA-4/FOXA IS A CRITICAL TRANSCRIPTION FACTOR REQUIRED FOR STARVATION RESPONSE

Chapter 3 is a manuscript in preparation for publication. The authors of this manuscript are Karyn Sheaffer, Trisha Brock and Susan Mango.

3.1 Abstract

Nutrients regulate whole body responses through the GI tract. Postembryonic development requires much energy and is regulated tightly by energy intake of organisms (Martos-Moreno et al., 2009). The gastrointestinal (GI) tract is the central player in nutrient intake; however, little is known about factors in the gut that regulate development (Magni et al., 2009). FoxA factors are master regulators of the GI tract during embryogenesis and after birth, but little is known about their regulation (Friedman and Kaestner, 2006). I use the simple digestive tract of *C. elegans* to investigate the role of *pha-4/FoxA* in whole organism responses to nutrients.

In *C. elegans*, starvation causes growth arrest at multiple stages in postembryonic development including L1 diapause, dauer and adult reproductive diapause. Diverse signaling pathways regulate these developmental arrests (Angelo and Van Gilst, 2009; Fukuyama et al., 2006; Riddle et al., 1981). It is unclear how starvation regulates proper developmental arrests in different stages. Proper developmental arrest during L1 diapause has been shown to be required for survival during periods of starvation (Baugh and Sternberg, 2006; Fukuyama et al., 2006; Henderson et al., 2006). However, little is known about factors that are required during L1 diapause and recovery.

In *C. elegans*, *pha-4/FoxA* has been shown to be the master regulator of foregut development as well as being critical for responses to nutrient intake after birth (Ao et al., 2004; Horner et al., 1998; Panowski et al., 2007; Sheaffer et al., 2008). Here I show that *pha-4/FoxA* is required for L1 diapause survival post

embryonically. I show that levels of PHA-4 are important for starvation survival; loss of *pha-4/FoxA* decreases survival and over expression of *pha-4/FoxA* increases starvation survival. To determine when *pha-4/FoxA* is required during starvation, I examined the L1 developmental arrest during starvation and recovery after starvation. *pha-4/FoxA* is required for initiation of development after periods of starvation and is not required for developmental arrest during starvation. To investigate transcriptional activity of PHA-4 during starvation, I observed the effect of starvation on PHA-4 during L1 and see no change in levels or localization. To determine how PHA-4 transcriptional activity changes in response to nutrients, we are using genomewide approaches to determine targets of *pha-4/FoxA* that are important for L1 starvation survival.

3.2 Introduction

3.2.1 Coordination of Nutrient Intake and Development

All organisms coordinate nutrient intake and growth but little is known about the mechanism of transcriptional regulation and the role of the gastrointestinal (GI) tract. Malnutrition, after birth, delays growth and reproductive development (Martos-Moreno et al., 2009). The GI tract plays a central role in food intake and relaying information about energy status throughout the body in mammals (Magni et al., 2009). Research in the GI tract and how it responds dynamically at the whole animal level is difficult in higher organisms. The digestive system in mammals is extremely complex with many organs required for multiple functions. We use the simple digestive tract configuration in *C. elegans* to

investigate the role of the gut in the regulation of postembryonic development by nutrients.

Starvation causes developmental arrests at multiple stages in postembryonic development including L1 diapause, dauer and adult reproductive diapause in *C. elegans* (Baugh and Sternberg, 2006; Cassada and Russell, 1975; Fukuyama et al., 2006; Riddle et al., 1981; Van Gilst et al., 2005b). Studies of L1 diapause have shown factors that are required for the proper developmental arrest in response to starvation (Baugh and Sternberg, 2006; Fukuyama et al., 2006; Henderson et al., 2006). However, little is known about how nutrient intake initiates development after periods of starvation. Here we investigate the role of *FoxA/pha-4,* a master regulator of the foregut, in the response to acute starvation during L1 diapause in *C. elegans.*

3.2.2 Starvation Response in *C. elegans*

Diverse signaling pathways differentially regulate developmental arrests in response to starvation in different stages. *C. elegans* postembryonic development consists of the progression through four larval stages until adulthood. First stage (L1) larvae, if placed into starvation immediately after hatching, will arrest development (Baugh and Sternberg, 2006; Fukuyama et al., 2006). Starvation, if initiated during the transition from the first to second larval stages, cause worms to form an alternate larval form called dauer (Cassada and Russell, 1975). Starvation encountered during early adulthood causes arrest in reproductive development

(Angelo and Van Gilst, 2009). It is unclear how starvation regulates proper developmental arrests in different stages.

Dauer larvae formation is the most well characterized developmental arrest in response to starvation in *C. elegans.* Dauers are characterized by morphological and behavioral changes. Dauer larvae shutdown feeding by various modifications including sealing the mouth closed, constriction of the pharynx and shutdown of pumping (Cassada and Russell, 1975; Riddle et al., 1981; Vowels and Thomas, 1992). The cuticle becomes thickened and produces ridges, called alae, for protection and movement (Cassada and Russell, 1975). Dauer larvae fully depend on the mobilization of lipids for energy (Wadsworth and Riddle, 1989). *C. elegans* in this state are viable for months and addition of food will promote the opening of the gut, feeding and normal growth into a reproductive adult (Klass and Hirsh, 1976).

Multiple genetic networks are required for the integration of environmental signals to regulate the dauer stage (Gottlieb and Ruvkun, 1994; Riddle et al., 1981). Environmental stress caused by lack of food, crowding conditions and temperature cause dauer formation (Golden and Riddle, 1984). Chemosensory neurons and intact neuronal signaling are required for both formation and recovery of dauer (Albert et al., 1981). Transforming growth factor beta (TGF-ß) (Patterson et al., 1997; Ren et al., 1996), insulin (Gems et al., 1998), cyclic guanosine monophosphate (cGMP) (Birnby et al., 2000) and nuclear hormone receptor (daf-12) signaling (Antebi et al., 2000; Motola et al., 2006) have all been shown to modulate dauer formation. Little is known how these signaling pathways contribute to starvation response during L1 and adult reproductive diapause.

First stage (L1) larvae, when subjected to starvation immediately after hatching, arrest development in L1 diapause. Survival during L1 diapause is relatively short (2-3 weeks) compared to the long lived dauer (approx. 6 months) (Klass and Hirsh, 1976). Both L1 and dauer larvae depend on lipid mobilization for energy, however they show differences in the control of metabolic programs at the transcriptional level. *C. elegans* use both gluconeogenesis and the glyoxlate pathways to generate carbohydrates from noncarbohydrate carbon sources and fat, respectively (Van Gilst et al., 2005b; Wadsworth and Riddle, 1989). Dauer larvae show robust increase in expression of gluconeogenesis enzymes (Wang and Kim, 2003), whereas L1 starvation has little effect on these same genes (Van Gilst et al., 2005b). The glyoxylate pathway, although generally increased in both L1 and dauer, is regulated differently between specific components at the gene expression level (Van Gilst et al., 2005b; Wang and Kim, 2003). These data suggest that different factors may be required for starvation transcriptional responses during L1 diapause and dauer.

The regulation of developmental arrest during L1 diapause is important for starvation survival. Improper arrest of cell divisions in L1 has been shown to decrease starvation survival in several insulin signaling mutants (Baugh and Sternberg, 2006; Fukuyama et al., 2006). *daf-16*/FoxO, a forkhead transcription factor, has been shown to be required for starvation survival by regulating genes required for development and cell cycle arrest (*lin-4* and *cki-1*) that are important for establishing growth arrest during L1 diapause (Baugh and Sternberg, 2006; Henderson et al., 2006).

Starvation survival is modulated through multiple other responses. Signaling through chemosensory neurons is required for sensing of food intake and coordinating responses throughout the body through insulin and MAP kinase signaling (Kang and Avery, 2008; Lee and Ashrafi, 2008; You et al., 2006). Recycling and reuse of cellular components is important during starvation and defects in protein repair and autophagy have decreased starvation survival (Gomez et al., 2007; Kang and Avery, 2009). Lipases, *fil-1* and *fil-2*, are required for lipid breakdown during starvation and are regulated at the RNA level (Jo et al., 2009). *nhr-49* and *mdt-1*, a nuclear hormone receptor and a mediator subunit respectively, are required for transcriptional activation of enzymes in fatty acid beta-oxidation and survival during L1 and adult reproductive diapause (Taubert et al., 2008; Taubert et al., 2006; Van Gilst et al., 2005a; Van Gilst et al., 2005b). These processes are important for starvation survival; however, it is unclear whether these same processes have a role in developmental arrests induced by starvation in other stages.

3.2.3 Growth Recovery After Periods of Starvation

A key part of starvation survival lies in the ability to recover and begin growth when food is reintroduced. This requires the ability to sense the availability of food, resume feeding, uptake nutrients into the intestinal cells and initiate developmental programs throughout the body. Failure to proceed out of the L1 arrest after hatching has been shown in several different kinds of mutants that are defective in feeding. *pha-4/FoxA* is required embryonically for pharynx

development (Horner et al., 1998). *pha-4* null mutants hatch with no pharynx, cannot feed and never proceed out of L1 arrest (Gaudet and Mango, 2002). Similarly, ceramide glucosyltransferase (CGT) mutants are constitutively L1 arrested due to feeding dysfunction that prevents bacteria from entering the intestine (Marza et al., 2009). Muscarinic signaling modulates MAP kinase activity in the pharynx, and if unregulated, leads to grinder dysfunction and failure to break open bacteria to release nutrients into the intestine (You et al., 2006). These worms fail to initiate robust feeding after starvation and do not survive periods of starvation. Dysfunction of nutrient absorption in the intestine also causes constitutive L1 arrest. A vacuolar ATPase, *vha-6*, is required for acidification of the intestine and uptake of dipeptides and mutants show L1 arrest (Allman et al., 2009). These data show that functional feeding and nutrient absorption through the gut is required for progression of growth through L1 diapause.

Initiation of growth programs is important for progression out of L1 diapause. Genomewide expression studies have identified 446 feeding response genes that change during feeding and exit out of L1 starvation (Wang and Kim, 2003). These genes are enriched in metabolism and growth processes (Wang and Kim, 2003). The transcriptional response to feeding during L1 diapause is fully activated in a shorter time period compared to the transcriptional response to starvation. Pausing of RNA Polymerase II at the promoters of genes important for growth may be required for quick activation of the developmental program after L1 diapause (Baugh et al., 2009). This suggests that genes are poised for transcriptional activation during recovery out of L1 arrest.

3.2.4 *pha-4/FoxA* is Important in Organism Responses to Nutrient Intake

FoxA factors have been shown to be important regulators of fasting response in mice. The gluconeogenic program is tightly regulated by food intake and requires transcriptional activators such as glucocorticoid receptor (GR) and cAMP response element binding (CREB) to coordinate expression of targets (Zhang et al., 2005). PEPCK, Tat and Igfbp-1 are all induced by starvation and require FoxA2 for induction (Wolfrum et al., 2003; Wolfrum and Stoffel, 2006). Loss of FoxA2 inhibits binding of GR and CREB to these targets during starvation (Zhang et al., 2005). FoxA factors are required for transcriptional responses to fasting by allowing binding of transcriptional activators induced by starvation to bind promoters and activate gene expression.

There are several lines of evidence that show that *pha-4/FoxA* is important in coupling nutrient intake and survival in *C. elegans*. We see that *pha-4* is required during organismal responses to both chronic starvation recovery and decreased nutrient signaling. Loss of *pha-4* during dauer recovery prohibits pharynx remodeling and feeding, leading to death (Ao et al., 2004). Secondly, *pha-4* is required for lifespan extension effects of dietary restriction. Restricting diet by either feeding mutations or access to *E. coli* leads to about a 20% increase in mean lifespan and *pha-4* is necessary for this increase (Panowski et al., 2007). Thirdly, the Target of Rapamycin (TOR) pathway negatively regulates *pha-4* activity. The TOR pathway is activated by nutrients and is required for growth and aging. *pha-4* is absolutely required for the increased lifespan due to reduced levels of TOR kinase (Sheaffer et al., 2008).

Here we investigate the role of *FoxA/pha-4,* a master regulator of the foregut, in the response to starvation during L1 diapause in *C. elegans*. We show that *pha-4/foxa* is a critical transcription factor during starvation response in *C. elegans. pha-4/FoxA* level is important post-embryonically for L1 starvation survival and is required for growth recovery after periods of starvation. Finally, we investigate the role of *pha-4/FoxA* in transcriptional starvation response.

3.3 Materials and Methods

3.3.1 Strains and Worm Growth

C. elegans strains were maintained at 20°C (Brenner, 1974) unless otherwise noted. Feeding bacterial strains used were OP50 (Brenner, 1974) and S. comamonas (Avery and Shtonda, 2003). Worm strains used were SM190: *smg-1(cc546ts); pha-4(zu225) V(Gaudet and Mango, 2002)*, PD8120: *smg-1(cc546ts*), SM1755: *N2* control strain backcrossed 4X to SM1754 , SM1754: *N2; stIs10389 (pha-4::GFP::3XFLAG)* backcrossed 4X (Zhong et al.), SS747: *bnIs1[pie-1::GFP::pgl-1 + unc-119(+)]*, SU93: *jcIs1[ajm-1::GFP+pRF4+unc-29(+) IV]*, SM1202: *cha-1(P1182); pxEx(pha-4::YFP, cha-1)* (Kiefer et al., 2007), SM1802: *pxIs28[pxEx114 (hs::pha-4; rol-6)]* integrated line from original *hs::pha-4* (pML422) strain (Horner et al., 1998)and backcrossed 8X, SM1746: *N2* control strain 8X backcrossed to SM1802.

We previously generated a temperature-sensitive configuration of *pha-4 (pha-4(ts))* by combining *pha-4(zu225)* with *smg-1(cc546ts*) (Gaudet and Mango, 2002). *pha-4(zu225)* contains a premature stop codon that renders *pha-4* mRNA subject to degradation by the nonsense-mediated decay (NMD) pathway

(Kaltenbach et al., 2005). *smg-1(cc546ts)* is a temperature-sensitive allele of the NMD component *smg-1* (www.addgene.org/labs/Fire/Andrew/Vec97.pdf). At 24° animals are viable whereas at 15° or 20°, animals die with reduced PHA-4 levels (Kaltenbach et al., 2005).

3.3.2 RNA Interference

Feeding RNAi protocol was performed according to (Sheaffer et al., 2008) with the following changes. HT115 bacteria (Timmons et al., 2001) expressing dsRNA for *mCherry* and *pha-4* was grown for 6 hours at 37°C and seeded on plates with 5 mM IPTG (MoleculA).

RNAi clones used were *pha-4* (bSEM865) (Kiefer et al., 2007) and *mCherry* (bSEM1098). The dsRNA plasmid for *mCherry* (bSEM1098) consists of a cloned 900 bp mCherry fragment using the following primers (attb-sites underlined, gene sequence bold): mCherry_ex1For_attb1: 5'-ggggACAAGTTTGTACAAAAAAGCAGGCTTC**atggtctcaaagggtgaagaagataacatg**-3', mCherry_ex4Rev_attb2: 5'-ggggACCACTTTGTACAAGAAAGCTGGGTC**ctacttatacaattcatccatgccacctg**-3'. The PCR product was inserted into pDonorT7 (Reddien et al., 2005) using a BP reaction (Invitrogen). The clone was transformed into *E. coli* strain HT115 (Timmons et al., 2001).

Soaking RNAi was performed according to (www.wormbook.org) with the following changes. RNA was made using Ampliscribe T7 Transcription Kit (Epicentre Biotechnologies). DNA template was amplified from *pha-4* RNAi vector

(bSEM865) using the following primers: Pha4 cdna reverse primer: 5'-TAATACGACTCACTATAGGGATCCAACATCCATCACGACC-3', T7: 5'-TAATACGACTCACTATAGG-3'. DNA template was amplified from *mCherry* RNAi vector (bSEM1098) using the following primers: cherry T7: 5'-TAATACGACTCACTATAGGCAGTTCGGTACTAACTAACCATA-3', T7: 5'-TAATACGACTCACTATAGG-3'.

After transcription, RNA was isolated using phenol/chloroform and ethanol precipitation. RNA was resuspended in RNase free water for 1-3 hours at 60°C. RNA was then checked for integrity by O.D. and agarose gel. Soaking RNAi experiments consisted of 800 µl (2 µg/µl RNA), 200 µl 5X RNAi Soaking Buffer (1.29X M9, 15 mM spermidine, 0.25% gelatin) and 200 µl (150 embryos/µl) were added to a 15 ml conical tube. Final concentrations were 1.33 µg/µl RNA and 25 embryos/µl. Well-fed hermaphrodites were incubated with alkaline hypochlorite to release embryos. Embryos were then washed four times with sterile water and added to soaking RNAi. Tubes were rotated on a nutator for duration of experiment at 20°C.

3.3.3 Starvation Survival Assays

Well-fed hermaphrodites were incubated with alkaline hypochlorite to release embryos. Embryos were then washed 4 times with sterile water. Embryos were placed into S basal (www.wormbook.org) and adjusted to a final concentration of 10 embryos/µl. Total volume never exceeded 3 ml in a 15 ml conical tube. Tubes were rotated on a nutator for duration of experiment at the appropriate

temperature. All experiments were carried out at 20°C except for the following exceptions. Experiments with the *pha-4 ts* and *smg-1* strains used worms that were hatched at 24°C, shifted to 15°C for the duration of starvation and allowed to recover at 24°C. Experiments with the *hs::pha-4* strain used worms that were grown and hatched at 15°C then shifted to 24°C for the duration of starvation and recovery. Embryos were allowed to hatch for 24 hours (Day 1) and triplicate samples of 30 µl each were taken at the appropriate times. Samples were placed onto plates with food and 2 days later number of worms past L1 were counted. Survival log rank tests were performed using GraphPad Prism.

3.3.4 Measuring PHA-4 Levels

For imaging, worms were picked onto an agar pad and anesthetized with 100 mM levamisole. To visualize PHA-4::YFP, images were captured at the same exposure with DeltaVision RT Deconvolution system and SoftWoRx software (Applied Precision). Nuclei of the pharynx and intestine were identified by DIC and measured for total intensity in Image J. Approximately 5-10 intestinal nuclei per worm were measured and averaged.

To visualize endogenous PHA-4, immunostaining was performed as described previously (Horner et al., 1998) using the anti-PHA-4 polyclonal antibody at 1:1000. Embryos were hatched onto plates with and without food and antibody stained 12 hours later. Images were captured with DeltaVision RT Deconvolution system and SoftWoRx software (Applied Precision).

3.3.5 Bead Feeding Assay

L1s were starved for either 3 or 7 days. Starved L1 larvae were placed on NGM plates with OP50, 200 mM sorbitol and 5 µL added of a 1% (vol/vol) solution of 0.5 µm polystyrene beads (Polysciences) (Fang-Yen et al., 2009). Samples of worms were taken at various times during recovery. For imaging, worms were picked onto an agar pad and anesthetized with 100 mM levamisole. Pictures were taken on a Zeiss AxioImager M2 upright compound microscope with Colibri light system and Apotome.

3.3.6 L1 Arrest and Recovery

L1s were starved for either 3 or 7 days. Starved L1 larvae were placed on NGM plates with OP50 and allowed to recover for appropriate times. For imaging, worms were picked onto an agar pad and anesthetized with 100mM levamisole. Pictures were taken on a Zeiss AxioImager M2 upright compound microscope with Colibri light system and Apotome.

3.3.7 RT-qPCR Analysis

L1s were starved 3 days and/or fed for 3 hours. RNA was harvested using Trizol Reagent (Invitrogen). RNA was then cleaned and concentrated using RNA clean and concentrator kit (Zymo Research). 500 ng RNA was used in RT reaction (Protoscript, NEB). One tenth of the RT reaction (0.1 µl) and SYBER green (BioRad) was used in a 20 µl PCR reaction run on a Mastercyler® ep realplex system (Eppendorf). Each sample was run in duplicate. M7.1 and *tbb-2* were previously

described to have no expression changes in starvation and were used as control genes (Brock et al., 2006).

3.4 Results

3.4.1 *pha-4/FoxA* Level is Important Postembryonically for L1 Starvation Survival

To investigate starvation response in *C. elegans* during L1 diapause, embryos were isolated and allowed to hatch in liquid containing no food. Twenty-four hours after embryo isolation was counted as Day 1 and used as our baseline to which we compare other time points. At various time points, samples were taken and placed on food. All samples were counted two days later for the number of worms that grew past the L1 stage. Normally wild type worm mean survival in this assay was approximately 10-15 days at 20°C.

Due to the requirement of *pha-4/FoxA* during response to dietary restriction and reduced nutrient signaling through the TOR pathway in adults (Panowski et al., 2007; Sheaffer et al., 2008), we hypothesized that *pha-4/FoxA* was important for acute starvation response in *C. elegans*. To reduce *pha-4* levels immediately after hatching, embryos were isolated and exposed to control and *pha-4* dsRNA in liquid without food. Using RT-qPCR we showed that this RNAi protocol caused approximately 4.5-fold reduction of *pha-4* RNA levels by the third day of starvation. We show that worms exposed to *pha-4(RNAi)* for 7 days of starvation have approximately 50% survival compared to our controls showing 80-90% survival (Figure 3.1A). We verified this finding by using the *pha-4; smg-1 ts (pha-4 ts)* temperature sensitive configuration strain (Kaltenbach et al., 2005). The

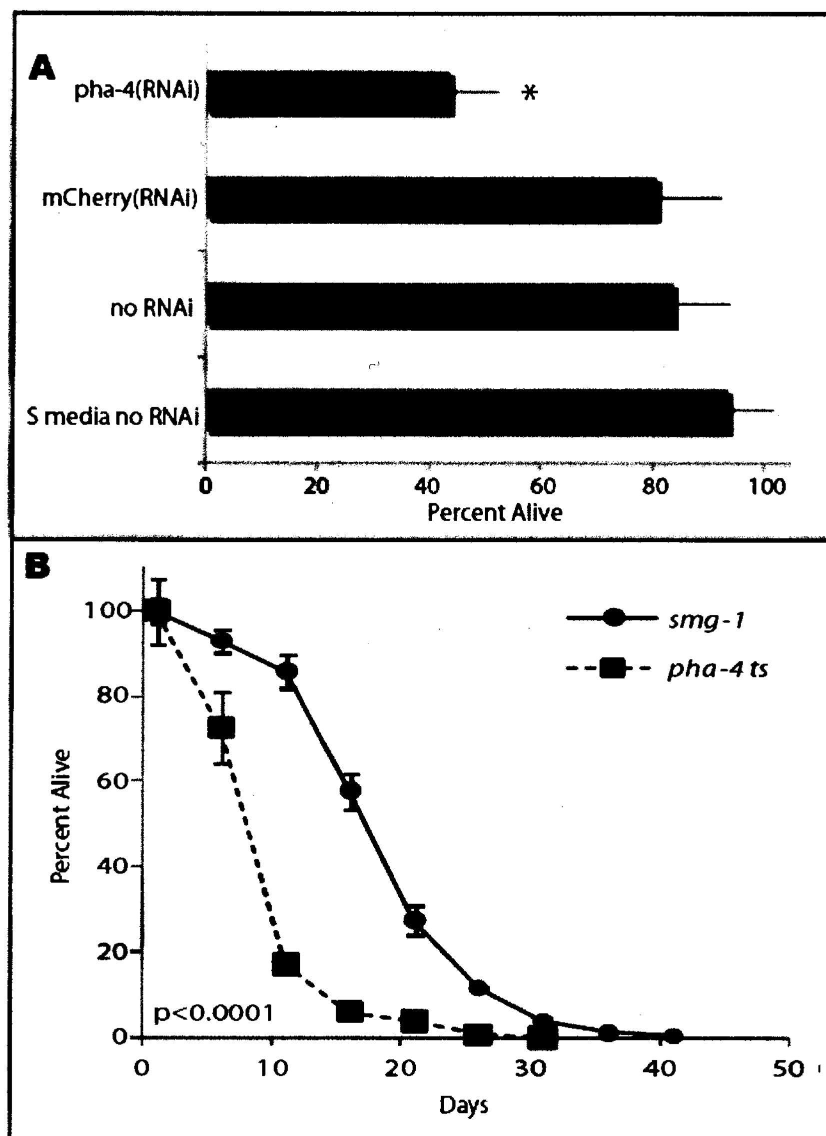

Figure 3.1. *pha-4* is required for L1 starvation survival.
A. Loss of *pha-4* greatly reduces starvation survival. Wild-type worms were exposed to soaking *mCherry(RNAi)*, *pha-4(RNAi)* and no treatment without food for 7 days. To determine viability, number of worms past L1 were counted after 2 days of recovery on food. Results are an average of three independent experiments, n=300-500 worms counted for each strain per experiment, error bars represent standard error, *p=0.038 t-test.
B. Loss of *pha-4* decreases starvation survival over time. Mean survival is decreased in *pha-4 ts* (11.05±0.47 days) compared to *smg-1* (20.1±0.7 days). To determine viability, triplicate samples of worms were taken each day and transferred to food at permissive temperature number of worms past L1 were counted after 2 days of recovery. Result shown is a representative experiment, n=200-650 worms counted for each strains per day, error bars represent standard error, p-value represents log rank (Mantel Cox) test.

temperature sensitive worms were grown at permissive temperature, embryos were isolated and allowed to hatch for 8 hours. L1s were then shifted to non-permissive temperature for the duration of starvation. Worms were then allowed to recover on food at permissive temperature. Using this method, we showed that reduced levels of *pha-4* caused severe reductions in both mean and maximum starvation survival (Figure 3.1B).

Because worms are extremely sensitive to the composition of liquid media to which they are exposed, we used two controls to validate our starvation survival results. To control for any possible effects due to the high concentration of RNA in our loss of function assays, we had a control that used water in replace of dsRNA (no RNA control). To control for any possible effects of cholesterol in our temperature sensitive assays, we starved worms in S media, which differs only in addition of cholesterol (S media control). Both of our controls showed no difference in starvation survival compare to *mCherry(RNAi)* at 7 days of starvation (Figure 3.1A).

Over-expression of PHA-4 during embryogenesis can induce extra pharyngeal cells however over-expression during aging is not sufficient for promoting lifespan (Horner et al., 1998; Panowski et al., 2007; Sheaffer et al., 2008). We wanted to test if *pha-4* is sufficient to promote L1 starvation survival. We used two different transgenic worm strains to ectopically express PHA-4 to test for starvation survival. PHA-4::GFP::3XFLAG is composed of the full-length genomic *pha-4* promoter and coding region with a C-terminal GFP and 3X FLAG tag (Zhong et al.). This construct has been shown to rescue the *pha-4(q490)* null allele (data not shown). Using RT-qPCR, we determined that this strain had 12±5 fold higher *pha-4*

RNA levels compared to wild-type L1. *hs::pha-4* contains a construct that uses the heat shock promoter to express PHA-4 in all cells (Horner et al., 1998). The heat shock promoter was induced at 25°C during the entire lifetime as well as duration of starvation assay. Both of these transgenic lines showed increased mean starvation survival (Figure 3.2A,B).

pha-4/FoxA is well known for its requirement in foregut development during embryogenesis (Horner et al., 1998). Our starvation assays were designed to bypass any embryonic effects by exposing worms to either RNAi or nonpermissive temperatures after completion of embryonic development. Previous studies have shown that loss of *pha-4* at 16E or the L1 stage, using the *pha-4 ts* strain, does not cause any defects in pharynx morphology (Gaudet and Mango, 2002; Kiefer et al., 2007). Using RNAi to reduce levels of *pha-4*, we showed that 100% of worms have normal pharynx morphology at three and seven days of starvation (Appendix B).

3.4.2 *pha-4* is required for Growth Recovery after Periods of Starvation

Our starvation survival assay tests both starvation survival and recovery. The most well characterized process that contributes to starvation survival is the establishment of developmental arrest during L1 diapause (Baugh and Sternberg, 2006; Fukuyama et al., 2006). Progression of growth through the L1 stage can be followed by observation of cell divisions. Mutants that show extra cell divisions during L1 diapause have decreased survival during L1 starvation (Baugh and Sternberg, 2006; Fukuyama et al., 2006). Both the seam cell lineage (V1-V6) and the

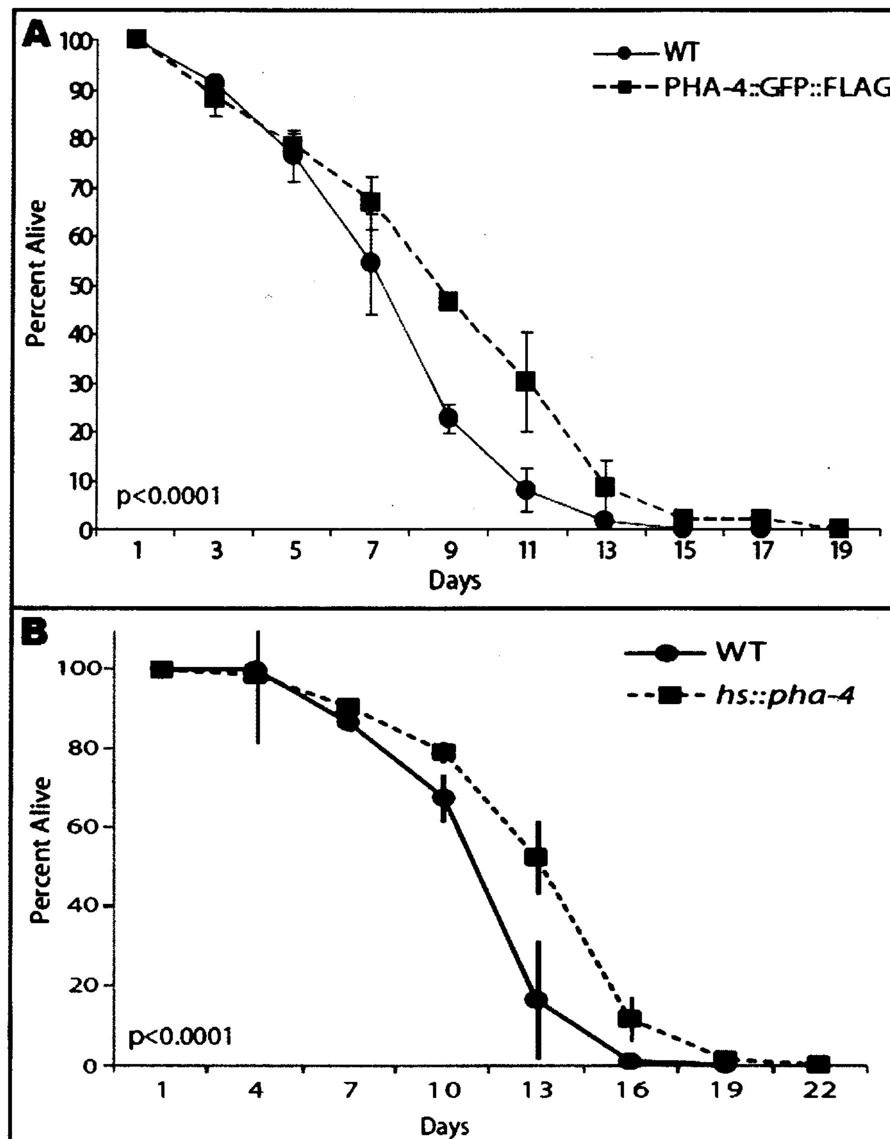

Figure 3.2. PHA-4 overexpression increases starvation survival.
A. Mean survival was increased in PHA-4::GFP::FLAG (9.4±0.24 days) compared to WT (8.3±0.19 days). PHA-4::GFP::FLAG and outcrossed WT worms were subjected to starvation at 20°C. To determine viability, number of worms past L1 were counted after 2 days of recovery on food. Results are an average of two independent experiments, n=500-1900 worms counted for each strain per experiment, error bars represent standard error, p-value represents log rank (Mantel Cox) test.
B. Mean survival was increased in *hs::pha-4* (13.96±0.37 days) compared to WT (12.1±0.28 days). *hs::pha-4* and outcrossed WT worms were subjected to starvation at 25°C for their entire lifetime. To determine viability, number of worms past L1 were counted after 2 days of recovery on food. Results are an average of two independent experiments, n=500-1900 worms counted for each strain per experiment, error bars represent standard error, p-value represents log rank (Mantel Cox) test.

primordial germ cells (Z2 and Z3), go through divisions in the developmental progression from L1 to L2 stage (Kimble and Crittenden, 2005). The L2 stage begins at the time all the seam cell lineages have gone through all divisions. To follow both germ and seam cells during L1 diapause recovery, we used the PGL-1::GFP and AJM-1::GFP reporters (Koppen et al., 2001; Updike and Strome, 2009). We starved *mCherry(RNAi)* and *pha-4(RNAi)* treated worms for 3 and 7 days and checked L1 arrest during starvation. Both treatments show 100% of worms were arrested completely in L1 during starvation, showing no divisions in either germ cells (Figure 3.3A) or seam cells (data not shown). These data suggest that *pha-4* is not required during starvation for establishment of developmental arrest.

To test for survival during starvation only, L1 larvae were exposed to control or *pha-4(RNAi)* for 7 days of starvation. Samples were then measured for viability by counting the number of worms that moved in response to touch. Worms from the same samples were then transferred to food and counted 2 days later for growth past L1. Compared to our controls, *pha-4(RNAi)* treatment showed similar L1 survival before recovery on food (Figure 3.3B). However after 2 days of recovery on food, we saw only 38±14% survival compared to 91±11% and 84±21% survival in our no RNA and *mCherry(RNAi)* controls, respectively (Figure 3.3B). This was a surprising result because our loss of function starvation assays in Figure 3.1 reduced *pha-4* by either RNAi or temperature shift during starvation only. There are two possible explanations of these conflicts in the requirement of *pha-4* during starvation. First, it is possible that we are not able to observe all dead larvae due to the dissolution of dead bodies in our liquid starvation assay (Figure 3.3B). Secondly,

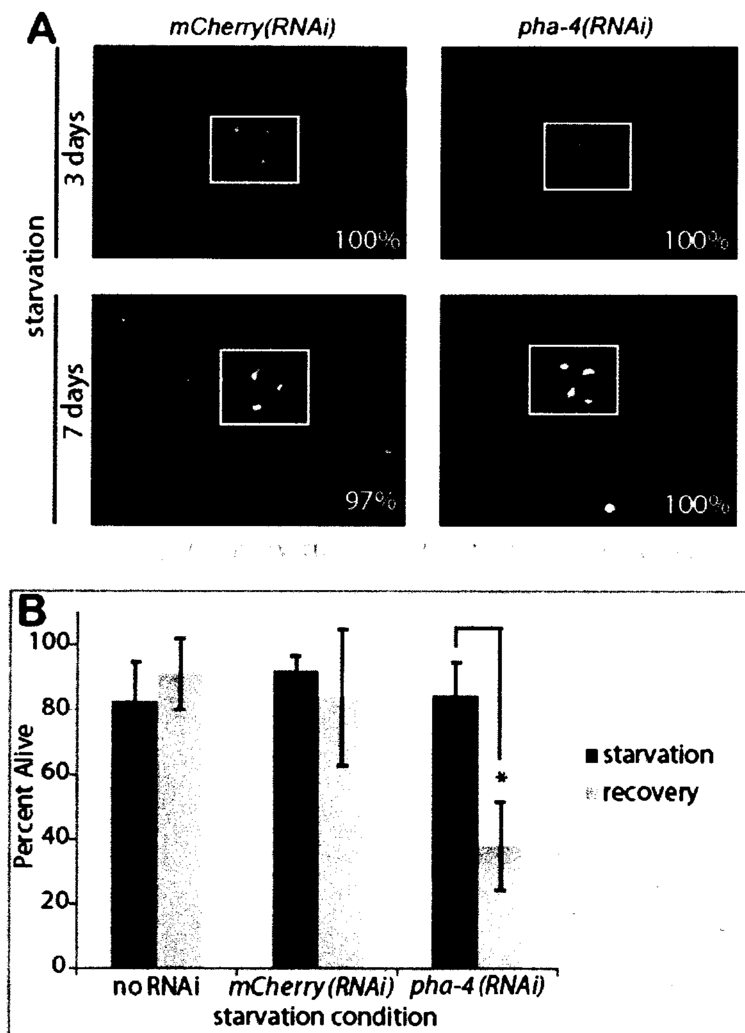

Figure 3.3. *pha-4* is not required for developmental arrest or survival during starvation.

A. Loss of *pha-4* has no effect on developmental arrest induced by starvation during L1. Wild-type worms were exposed to *mCherry(RNAi)* or *pha-4(RNAi)* without food for 3 or 7 days. PGL-1::GFP was used to visualize germ cells in starved L1s. L1 stage was determined by number of germ cells with GFP expression. Representative images and percentage of worms in L1 are shown above. Boxes mark germ cells, results represent one experiment, n=24-78 worms counted for each condition per time point.

B. Loss of *pha-4* reduces recovery from starvation not survival during starvation. Wild-type worms were exposed to *mCherry(RNAi)*, *pha-4(RNAi)* or no treatment without food for 7-9 days. To determine survival during starvation, one sample was taken and L1s were scored as viable based on movement in response to touch. To determine survival during recovery, number of worms past L1 were counted after 2 days on food. Results are an average of two independent experiments, n=50-200 worms counted for each condition per experiment, error bars represent standard error, *p=0.038 t-test.

pha-4 may be required at the onset of recovery and worms in our loss of function starvation assays would have very reduced levels of *pha-4* at the initial stages of recovery (Figure 3.1).

Because we did not observe any defects due to loss of *pha-4* during starvation, we tested the requirement of *pha-4* during starvation recovery. We starved wild type worms for various times and then allowed them to recover on control or *pha-4(RNAi).* Recovery from 13 days of starvation was unaffected by *pha-4(RNAi)* treatment with 67.9±15.7% survival compared to 56±8.8% survival in the control. However, reduction of protein levels by RNAi may not have been fast enough to reduce PHA-4 levels to affect recovery. We are currently measuring how quickly PHA-4 protein levels are reduced by our RNAi protocols.

We wanted to further characterize recovery defects due to *pha-4(RNAi)* treatment. Using the PGL-1::GFP reporter, we followed recovery out of L1 diapause by observing germ cell divisions (Updike and Strome, 2009). *mCherry(RNAi)* and *pha-4(RNAi)* worms were starved for either three or seven days and then allowed to recover on food for specific times. After 3 days of starvation we saw that *pha-4(RNAi)* caused slower progression of germ cell divisions at 12 hours after feeding (36% *pha-4(RNAi)* compared to 66% *mCherry(RNAi)*) (Figure 3.4). However after 24 hours of recovery, we saw no difference in L1 arrest between pha-4(RNAi) and control showing developmental progression in 95% and 100%, respectively. This is interesting because survival after 3 days of starvation was 100%. After 7 days of starvation, recovery in *pha-4(RNAi)* worms was significantly blocked. Only 59% of *pha-4(RNAi)* treated worms had germ cell divisions at 24 hours compared to 93%

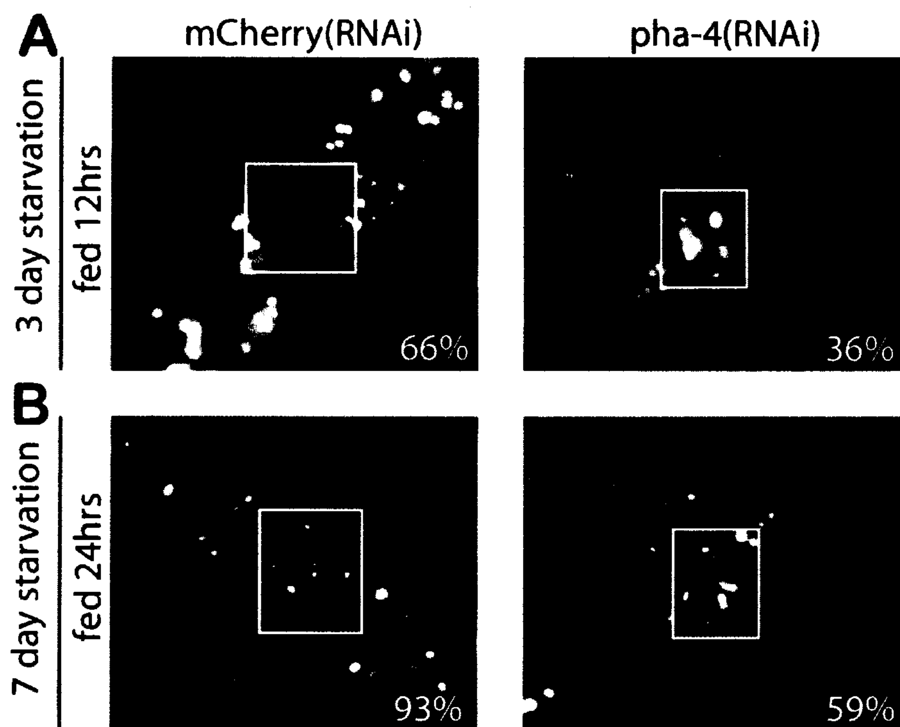

Figure 3.4. *pha-4* is required for developmental recovery after starvation.
A. Loss of *pha-4* delays developmental recovery after 3 days of starvation. Wild-type worms were exposed to *mCherry(RNAi)* or *pha-4(RNAi)* without food for 3 days and then recovered on food. PGL-1::GFP was used to visualize germ cells. At 12 hours of recovery, 66% of *mCherry(RNAi)* treated worms have germ cell divisions compared to 36% of *pha-4(RNAi)* treated worms. Representative images and percentage of worms with cell divisions is shown above. Boxes mark germ cells, results represent one experiment, n=24-78 worms counted for each condition per time point.
B. Loss of *pha-4* inhibits developmental recovery after 7 days of starvation. Wild-type worms were exposed to *mCherry(RNAi)* or *pha-4(RNAi)* without food for 7 days and then recovered on food. PGL-1::GFP was used to visualize germ cells. At 24 hours of recovery, 93% of *mCherry(RNAi)* treated worms have germ cell divisions compared to 59% of *pha-4(RNAi)* treated worms. Representative images and percentage of worms with cell divisions is shown above. Boxes mark germ cells, results represent one experiment, n=24-78 worms counted for each condition per time point.

of *mCherry(RNAi)* (Figure 3.4). This defect in L1 recovery corresponded closely with the 44±9% starvation survival observed in *pha-4(RNAi)* treated worms (Figure 3.1A). These data suggest that *pha-4* is required for recovery of growth through L1 after periods of starvation.

Smaller size bacteria, such as *S. comamonas,* exacerbate pharynx malfunctions, specifically grinder defects (You et al., 2006). We used the *E.coli* strains *OP50* (Brenner, 1974) and *HT115* (Timmons et al., 2001) containing RNAi constructs as well as *S. comamonas* (Avery and Shtonda, 2003) to test for pharynx malfunctions due to loss of *pha-4.* We starved *pha-4(RNAi)* and control treated worms for 9 days and then fed worms different bacteria and counted how many grew past L1. We saw that starvation recovery on different types of bacteria had no effect on control survival (Appendix B). *pha-4(RNAi)* caused decreased survival compared to controls but recovery on different bacteria showed no significant change in *pha-4(RNAi)* effects (Appendix B). These data suggest that *pha-4(RNAi)* does not cause grinder defects however these experiments do not test more general feeding defects.

We tested for the requirement of *pha-4* in the initiation of feeding during starvation recovery. During L1 starvation, pumping is maintained at a basal rate, and this rate is important for ingestion of food during recovery from starvation (Kang et al., 2007; You et al., 2006). To measure feeding dynamics, we mixed fluorescent 0.5 μm beads into bacteria, starved *mCherry(RNAi)* and *pha-4(RNAi)* worms for 3 and 7 days and allowed worms to recovered on a mix of beads and bacteria for various times. Worms were scored for beads in the pharynx only or

both pharynx and intestine. The failure of beads to progress into the intestine would suggest a failure of pharynx function whereas presence of beads in the intestine would suggest functional pumping through the pharynx. Feeding after three days of starvation resumed completely in 2 hours with 100% worms exposed to either treatment showing beads in both the pharynx and intestine (Appendix B). Seven days of starvation caused longer feeding recovery time with intestinal beads in only 50% of control worms after 2 hours of feeding. However, after 10-12 hours of feeding 74% of control worms had beads in the intestine compared to 45% of the *pha-4(RNAi)* worms. These data corresponded closely with the 44±9% survival rate seen with *pha-4(RNAi)* treatment (Figure 3.1A). These data suggest that loss of *pha-4* causes feeding defects that may be required for ingestion of food and growth out of L1 arrest.

3.4.3 Investigation of the Role of *pha-4* in Transcriptional Starvation Response

Progression out of L1 diapause also requires the transcriptional activation of genes important in growth and development (Baugh et al., 2009). *pha-4* is important for the transcriptional activation of many genes critical for the development of the pharynx (Gaudet and Mango, 2002; Gaudet et al., 2004; Kiefer et al., 2007; Updike and Mango, 2006). We sought to find out if *pha-4* is required for genes that are activated in L1 starvation and recovery.

PHA-4 is expressed throughout the entire gut making it a good candidate for transcriptional regulation by nutrient intake. We first tested if protein levels or

localization was affected by starvation in L1 larvae. A PHA-4::YFP full-length protein fusion was used to observe expression in L1 larvae either fed or starved for different times. There was no change in expression in either the pharynx or intestine at 12 and 36 hours of starvation (Appendix B). There were no changes in levels during shorter periods of starvation in the intestine (Appendix B). PHA-4 expression is found in the nucleus in both the pharynx and intestine during all conditions (Appendix B). Similar findings were shown by immunohistochemistry using an antibody to detect endogenous PHA-4 (Appendix B). These data are similar to previous reports that find that nutrients have no effect on PHA-4 levels or localization in the adult (Panowski et al., 2007; Sheaffer et al., 2008).

We are currently taking a whole genome wide approach to determine if starvation response genes are regulated by *pha-4*. We are performing microarrays to find genes differentially expressed during starvation and recovery that require *pha-4*. We will compare our microarray datasets to those genes that have promoters that are bound by PHA-4 (Zhong et al.). These genes will be potential targets of *pha-4* that modulate starvation survival.

3.5 Discussion

3.5.1 *pha-4/FoxA* is Important for Whole Organism Responses to Nutrient Intake

pha-4/FoxA is required for whole organism responses to nutrient intake (Ao et al., 2004; Panowski et al., 2007; Sheaffer et al., 2008). We have shown that *pha-4/FoxA* is required for L1 starvation survival postembryonically. This supports data in mice and shows FoxA factors have a conserved role in fasting response (Wolfrum

et al., 2004; Zhang et al., 2005). All FoxA studies in mice have been limited to reducing *pha-4* levels in individual tissues to avoid embryonic death. We have designed a starvation assay that specifically reduces *pha-4* levels in the whole organism after birth to rule out any developmental complications.

pha-4/FoxA is not required for growth arrest induced by starvation. An important aspect of starvation survival has been shown to be the arrest of cell divisions (Baugh and Sternberg, 2006; Fukuyama et al., 2006). Insulin signaling through *daf-16/FoxO* has been shown to play an important role in this growth arrest (Baugh and Sternberg, 2006). We find that *pha-4/FoxA* has no effect on L1 arrest during starvation. This suggests that *pha-4/FoxA* is acting independently of Insulin signaling. This is further supported by lifespan studies that show *pha-4/FoxA* has no effect on lifespan extension due to decreased Insulin signaling (Panowski et al., 2007; Sheaffer et al., 2008). These data shed insight on the debate about the mechanism of regulation of *pha-4/FoxA* and will be discussed later.

pha-4/FoxA may be required during starvation. We show that *pha-4(RNAi)* treatment during starvation has no effect on L1 survival after 7 days. Decreased recovery normally is seen before death during starvation. Other studies that measure survival of L1 larvae during starvation use later time points than we use (Baugh and Sternberg, 2006). These data suggest that our experimental design may miss some kind of starvation defect.

Growth initiation after periods of starvation requires *pha-4/FoxA*. We show that *pha-4(RNAi)* treatment during starvation blocks growth recovery after periods of starvation. These data suggest that *pha-4* has a role in starvation recovery.

However *pha-4(RNAi)* treatment during recovery has no effect on survival. This is probably due to either a delay in loss of PHA-4 protein levels by RNAi or may reflect a small window of time in which PHA-4 levels are critical. Preliminary tests suggest that PHA-4 levels are not reduced within the critical 12-24 hours in which development is reinitiated. We may be able to use the *pha-4 ts* strain to achieve a quicker loss of functional PHA-4 protein during recovery.

pha-4/FoxA regulates gut function in response to nutrients. We show that *pha-4* is required for efficient feeding. Our data support the recent findings in mice that show hypothalamic FoxA2 has a role in feeding behavior (Silva et al., 2009). It is unclear whether the feeding defect is causing the delay in growth progression. More sensitive tests for feeding, like measuring pumping rates, need to be done to investigate the role of *pha-4* in feeding. Other gut functions, such as nutrient absorption, that have been shown to be important for progression through L1 have not been tested for *pha-4* requirement (Allman et al., 2009).

3.5.2 Regulation of *pha-4/FoxA* Activity by Nutrients

There is strong evidence to show that nutrient intake responses require FoxA factors, but how FoxA factor transcriptional activity changes in response to nutrients is still very unclear. In the mouse liver, insulin signaling through AKT has been shown to phosphorylate FoxA2, which causes export out of the nucleus (Wolfrum et al., 2004). However, other groups have shown that FoxA2 in the liver is constitutively in the nucleus under the same conditions (Zhang et al., 2005). In *C. elegans*, PHA-4 protein levels do not change during low nutrient signaling conditions

such as dietary restriction or reduced TOR signaling (Panowski et al., 2007; Sheaffer et al., 2008). We show that PHA-4 levels and localization do not change in response to starvation in *C. elegans*. These data support the idea that PHA-4 transcriptional activity is not regulated by localization changes.

Transcriptional activity of FoxA factors may be modulated by modifications. In mice, several groups have shown phosphorylation of FoxA factors by kinases involved in nutrient sensing pathways (Nock et al., 2009; Wolfrum et al., 2003). It would be interesting to investigate if PHA-4 is post-transcriptionally modified in response to nutrients in *C. elegans*. One interesting genetic interaction has shown that the TOR pathway regulates *pha-4/FoxA* activity during lifespan in *C. elegans*. Specifically *rsks-1/S6K*, a well known kinase that is regulated by the TOR pathway in other organisms, was shown to negatively regulate *pha-4/FoxA* (Sheaffer et al., 2008). The interaction between RSKS-1 and PHA-4 would be interesting to test biochemically.

3.5.3 Transcriptional Response to Starvation

Our study has shown that there are two important phases of starvation survival, starvation and recovery. During starvation, metabolic, growth arrest and stress programs are initiated (Van Gilst et al., 2005b; Wang and Kim, 2003). However, during recovery, feeding also induces the expression of a similar number of genes of which are involved in development and growth (Baugh et al., 2009; Wang and Kim, 2003). These data suggest that both starvation and recovery depend heavily on transcriptional regulation. Interestingly, PHA-4 is bound to the

promoters of many of these genes that are induced by both starvation and feeding (T. Brock, pers. comm.). It is possible that PHA-4 could be playing a role in both phases of starvation survival.

Binding site affinity and PHA-4 level play major roles in the temporal activation of embryonic targets (Gaudet and Mango, 2002; Horner et al., 1998). Promoters containing high affinity sites are activated earlier compared to low affinity sites, which activate at later time points in development (Gaudet and Mango, 2002). Sites in promoters bound by PHA-4 in starved L1 larvae have a lower affinity consensus site compared to sites bound by PHA-4 in embryos (Zhong et al.). However, PHA-4 protein levels do not change appreciatory during L1 starvation. This suggests that PHA-4 protein may be modified and/or associated with co-factors that assist in the binding to sub par consensus sites and/or activation of transcription.

pha-4/FoxA plays an important role in organismal survival. It is interesting to note that many of gene promoters that are bound by PHA-4 and are increased in starvation are also implicated in aging (T. Brock, pers. comm.). *pha-4/FoxA* is required for normal lifespan (Panowski et al., 2007; Sheaffer et al., 2008). FoxA factors are required in a concentration dependent manner for development of the foregut (Horner et al., 1998; Mango et al., 1994). However over-expression of PHA-4 has no effect on lifespan whereas we see increased survival during starvation (Panowski et al., 2007; Sheaffer et al., 2008). This suggests that there are different survival responses that control starvation survival and adult aging. It would be

informative to investigate the possibility that *pha-4* is important for the regulation of the same target genes in both L1 starvation recovery and aging.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

My thesis work has investigated how nutrient intake and sensing occurs through the GI tract and causes whole organism responses during aging and development. In Chapter 2, I showed evidence of a previously unknown genetic interaction between the Target of Rapamycin (TOR) pathway and *pha-4/FoxA*. On the one hand, these data give insight into the transcriptional outputs of the TOR pathway through *pha-4/FoxA*. On the other hand, these data add more detail to the ongoing debate about the mechanism of nutrient regulation of *pha-4/FoxA*. In Chapter 3, I described the first evidence of *pha-4/FoxA* requirement during starvation response in *C. elegans*. These data show the importance of transcriptional regulation in the GI tract by *pha-4/FoxA* in response to nutrients during recovery from periods of starvation.

4.2 Outputs of the Target of Rapamycin (TOR) Pathway

4.2.1 Nutrient Sensing through the TOR Pathway

Target of Rapamycin (TOR) kinase has a conserved role in coupling nutrient status to growth in the cell. TOR kinase is found in two functionally diverse complexes that are differentially regulated by nutrient signaling (Laplante and Sabatini, 2009). TORC1 is activated by amino acids and growth factors and is required for growth through protein synthesis (Wullschleger et al., 2006). The TORC1 complex requires an adaptor protein, Raptor, for targeting to downstream targets and can be deactivated by rapamycin, which blocks interaction of Raptor with the complex (Guertin and Sabatini, 2007; Hara et al., 2002; Kim et al., 2002).

On the other hand, the TORC2 complex requires another partner called Rictor and is insensitive to amino acids and rapamycin (Sarbassov et al., 2004). TORC2 regulates multiple other processes including actin cytoskeletal reorganization (Jacinto et al., 2004).

TORC1 is the master sensor of energy status of the cells and coordinates growth and cell survival. The TOR pathway promotes translation initiation and translation, two processes required for growth. Direct phosphorylation of 4E-BP and S6 Kinase by TOR kinase causes increased translation initiation and translation, respectively (Wullschleger et al., 2006). In mechanisms that are less clear, TORC1 is also involved in the activation of transcriptional responses required for response to nutrients (Lempiainen et al., 2009; Marion et al., 2004; Martin et al., 2004). TORC1 is also a regulator of cell survival in part through autophagy, in which cellular components are degraded in response to inadequate cellular energy intake (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). Multiple effectors of TOR signaling have been characterized, but the full spectrum of TORC1 targets is still unknown.

TOR signaling is also a conserved regulator of aging. Mutations in TOR pathway genes have been shown to increase lifespan in simple organisms such as *S. cerevisiae, D. melangastor* and *C. elegans* (Jia et al., 2004; Kapahi et al., 2004; Powers et al., 2006; Vellai et al., 2003). Recent work in mice has shown that these effects on aging are conserved in higher organisms. Mice treated with rapamycin showed an increase in mean lifespan (Harrison et al., 2009). Aging regulation in mammals may be due to TOR pathway control of translation through S6 Kinase (Selman et al.,

2009). However, genetic studies in *C. elegans* suggest that translational regulation of lifespan is controlled by multiple transcriptional factors (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007).

The TOR pathway is required for growth and aging in *C. elegans*. Many of the TOR components have been shown to be conserved in worms and have defects in metabolism and growth (Honjoh et al., 2009; Jia et al., 2004; Jones et al., 2009; Long et al., 2002; Soukas et al., 2009). Mutations in TORC1, *let-363*/TOR kinase and *daf-15*/Raptor, have severe effects on postembryonic growth, protein synthesis, lipid storage, autophagy and aging (Jia et al., 2004; Long et al., 2002; Vellai et al., 2003). The positive regulator of TORC1, *rheb-1*/Rheb also shows severe growth defects (Honjoh et al., 2009). Homologs of many of the components of the TOR signaling network found in mammals have been found in worms but there is no biochemical evidence that has shown that these proteins interact *in vivo* or any evidence that TOR controls similar downstream targets as mammals.

4.2.2 Control of Translation and Ribosome Biogenesis by TOR Signaling

TOR signaling is required for translational control through several processes. Known mammalian downstream effectors of TOR kinase, S6 Kinase and 4EBP-1, are important regulators of translation. TOR signaling in yeast has been shown to be the master regulator of ribosome biogenesis by coordinating expression of ribosomal RNA and protein genes (Lempiainen et al., 2009; Marion et al., 2004; Martin et al., 2004). Only recently has work been done to validate that TOR signaling functions similarly in worms.

TOR signaling in worms partially recapitulates what has been seen in mammals. Both *let-363*/TOR and its mammalian target *rsks-1*/S6K are important for normal rates of protein synthesis in worms (Hansen et al., 2007; Panowski et al., 2007; Sheaffer et al., 2008). *rsks-1*/S6K has very well conserved phosphorylation sites in the catalytic domain; however, direct interactions between kinases that are known to phosphorylate S6 Kinase in other organisms, such as TOR and PDK, have not been tested in worms. However, there is no clear homolog for 4EBP-1 in worms (Long et al., 2002). Loss of any of the five eIF4E factors cause decreased protein synthesis rates (Syntichaki et al., 2007). How these initiation factors may function is still unclear and genetic studies have suggested that they may not function in TOR signaling in aging (Hansen et al., 2007; Sheaffer et al., 2008; Syntichaki et al., 2007).

My work provides more insight on how TOR signaling controls translation through ribosome biogenesis in *C. elegans*. Chapter 2 shows evidence that *let-363*/TOR and *ruvb-1*/RVB1 are critical for box C/D snoRNP complex localization and function. These data are the first example of the TOR pathway regulating ribosome biogenesis through the Box C/D snoRNPs. However, targets of TOR, *ife-2*/eIF4E and *rsks-1*/S6K, are not required for the localization of the complex. These data suggest that *let-363*/TOR may interact independently with *ruvb-1*/RVB1 to modify the snoRNP complex. To prove this genetic interaction is a bona fide target of TOR signaling, further work needs to be done to biochemically test the interactions of the TOR complex, *ruvb-1*/RVB1 and the Box C/D snoRNP complex.

4.2.3 Transcriptional Regulation by TOR Signaling during Lifespan Regulation

There are three lines of evidence that TOR signaling has a role in transcriptional regulation. In both yeast and mammals loss of TOR causes massive transcriptional changes (Cardenas et al., 1999; Guertin et al., 2006; Mahajan, 1994; Powers and Walter, 1999). Second, there is evidence that direct interaction of TOR kinase with transcription factors affects their activity. In yeast, TOR kinase regulates ribosomal protein gene expression by phosphorylation of FHL1 and Sfp1 (Lempiainen et al., 2009; Marion et al., 2004; Martin et al., 2004). Third, TOR kinase is found localized in the nucleus and bound to rDNA promoters in both yeast and mammals (Wei et al., 2009b; Wei and Zheng, 2009).

TOR signaling has been shown to play a role in the transcriptional response to dietary restriction in yeast (Medvedik et al., 2007; Wei et al., 2009a). TOR signaling has also been implicated in lifespan extension due to dietary restriction in *C. elegans*. Treating worms by restricting food and reducing TOR shows no additive increase in lifespan suggesting that both methods work in a similar fashion. However *eat-2* mutants sometimes show increased lifespan when TOR levels are reduced (Hansen et al., 2007; Henderson et al., 2006). Other components of TOR signaling have also been implicated in lifespan extension due to different modes of dietary restriction (Chen et al., 2009; Honjoh et al., 2009).

Chapter 2 provides evidence that loss of TOR signaling increases lifespan in a similar manner to DR. I showed that *pha-4/FoxA*, previously found to be required for DR (Panowski et al., 2007), is required for lifespan extension due to loss of TOR signaling. Reduced TOR inhibits translation but not all translation inhibition works

in a similar manner to control lifespan (Hansen et al., 2007). Targets of TORC1, *rsks-1*/S6K and *ife-2*/eIF4E, are known for lower levels of translation and increased lifespan (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). I showed that *rsks-1/S6K* absolutely requires *pha-4* for lifespan extension whereas *ife-2*/eIF4E does not. Recently *rsks-1/S6K* has been implicated further as an important regulator of DR response (Chen et al., 2009). These data suggest that dietary restriction responses are modulated through TOR signaling.

These data demonstrated for the first time genetic epitasis between the TOR pathway and a FoxA factor. My data support the idea that TOR signaling through *rsks-1/S6K* may directly modify *pha-4/FoxA*. Phosphorylation of FoxA factors in mammals may be important for transcriptional regulation (Howell and Stoffel, 2009; Wolfrum et al., 2004). It would be extremely interesting to test whether *rsks-1/ S6K* could phosphorylate *pha-4/FoxA* biochemically and if this modification could regulate transcriptional activation.

4.3 Regulation of *pha-4/FoxA* by Nutrients

This work and multiple studies have shown that FoxA factors have a conserved role in organismal response to nutrient status however, the regulation of FoxA factors by nutrients is still unclear. Addition of insulin or Akt phosphorylation has been shown to cause nuclear exclusion and inactivation of FoxA2 in Hep2 cells (Wolfrum et al., 2003) while starvation causes FoxA2 to be localized to the nucleus with no change in protein levels (Wolfrum et al., 2004). However, another group has shown FoxA2 constitutively in the nucleus of the liver using similar conditions

mentioned above (Zhang et al., 2005). Many FoxA2 metabolic targets show constitutive binding to promoters including Igfbp-1, PEPCK and TAT suggesting that even if FoxA is being shuttled out of the nucleus, there is no effect on its role in binding of target promoters (Wolfrum et al., 2003; Zhang et al., 2005). These seemingly contradictory results prompted us to investigate PHA-4/FoxA in *C. elegans*.

In my thesis work, *pha-4/FoxA* protein levels and localization were characterized during both aging and starvation. I show that during aging in the adult PHA-4 protein does not change localization or levels in response to reduced TOR signaling (Appendix A). I also determine that there is no change in PHA-4 protein during L1 starvation (Appendix B). Further using chromatin IP against PHA-4, we see that the majority of PHA-4 binds to promoters both during starvation and feeding (Appendix B). These data support the model that the transcriptional activity of *pha-4/FoxA* is not being regulated by transcription or localization.

I have also shown in Chapter 2 that *pha-4*/FoxA is regulated by the TOR pathway and acts independently of insulin signaling in *C. elegans*. Mutations in *let-363*/TOR and *rsks-1*/S6K mutants not *daf-2*/IR show suppression of *pha-4* lethality. These data show genetically TORC1 negatively regulates *pha-4/*FoxA activity. *pha-4*/FoxA is not required for lifespan extension due to reduction of insulin signaling. These data support the model that nutrients regulate *pha-4/FoxA* independently of insulin signaling. However, studies of PHA-4 protein levels, localization and modifications have not been done in insulin signaling mutants.

4.4 Transcriptional Response to Starvation

4.4.1 Characterization of Starvation Response in *C. elegans*

Starvation transcriptional response in *C. elegans* is very complex due to different outcomes when starvation is encountered at different developmental stages. Starved worms arrest in specific stages such as L1, dauer and adult diapause (Angelo and Van Gilst, 2009; Baugh and Sternberg, 2006; Fukuyama et al., 2006). Multiple signaling pathways dynamically regulate growth in response to nutrients (Grewal, 2009). Transcriptional competence is required for the activation of genes required for starvation survival as well as recovery from starvation (Baugh et al., 2009; Wang and Kim, 2003).

Chapter 3 provides evidence that there are two phases of starvation survival that show activation of gene expression. During starvation, although there is massive shutdown of growth and development, we see many genes involved in metabolism and stress response up regulated (Chapter 3). On the other hand, during starvation recovery, we see about the same number of genes activated. Starvation recovery genes are highly enriched for functions in growth and development (Chapter 3) (Baugh et al., 2009; Wang and Kim, 2003). These data suggest that there are transcriptional regulators that are required for coordination of gene expression changes genomewide during starvation response. Whole genome wide screens could be performed in *C. elegans* to search for these master regulators.

4.4.2 FoxA Factor Requirement in Starvation Response

There are still questions about how *pha-4*/FoxA controls organismal survival though the GI tract in response to nutrients. In mice FoxA factors are required for several metabolic programs regulated by starvation in the liver including gluconeogenesis and lipid metabolism (Wolfrum et al., 2003; Wolfrum and Stoffel, 2006; Zhang et al., 2005). However, work in mice has been limited to loss of FoxA function in individual tissues. In *C. elegans*, we have designed assays in which we can avoid developmental complications by reduction of *pha-4/FoxA* after birth in the entire organism.

An outstanding question from my research is what responses require *pha-4/FoxA* for organismal survival. Chapter 3 shows that *pha-4/FoxA* is required for growth recovery after periods of starvation in *C. elegans*. I show that PHA-4 is bound to the promoters of many genes that change expression during starvation. However, these genes do not require PHA-4 for expression changes or for starvation survival. Recent studies have shown that *pha-4/FoxA* is required for some SOD expression (Panowski et al., 2007) and is required for the increase in autophagy due to DR (Hansen et al., 2007). However, these studies do not show biochemical evidence of PHA-4 binding to promoters of these genes or that either of these targets is sufficient for increased survival. These data suggest PHA-4 may regulate multiple responses that together control multiple aspects of survival.

4.5 Conclusions

These data show the importance of transcriptional regulation in the GI tract by *pha-4/FoxA* in response to nutrients during recovery from periods of starvation. Future research needs to be done to determine whether other organisms possess a genetic interaction between the Target of Rapamycin (TOR) pathway and *pha-4/FoxA* and the nature of this interaction. It would also be very exciting to determine what genes are regulated by *pha-4/FoxA* during starvation response in *C. elegans.* My work has given us more insights on how nutrient intake and sensing occurs through the GI tract and causes whole organism responses during aging and development.

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APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Reprinted with permission from Sheaffer, K.L., Updike, D.L., and Mango, S.E. (2008). The Target of Rapamycin pathway antagonizes pha-4/FoxA to control development and aging. Curr Biol *18*, 1355-1364.

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Supplemental Data

The Target of Rapamycin Pathway Antagonizes *pha-4/FoxA* to Control Development and Aging

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Supplemental Experimental Procedures

Strains and Worm Growth

C. elegans strains were maintained at 20° [1] unless otherwise noted. We used SM1406: *unc-42(e279) ruvb-1(px34)/ evl-1(ar115)V* [2], SM190: *smg-1(cc546ts) I; pha-4(zu225) V* [3], SM1237: *dpy-11(e224) unc-42(e270) ruvb-1(px34)/evl-1(ar115) V* [2], RB1206: *rsks-1(ok1255) III* [4] was backcrossed six times against wild-type N2 (Bristol) for lifespan analysis, DR2381: *let-363(h111)/ dpy-5(e61) I* (kindly provided by Don Riddle [5]), KR441: *let-363(h111) dpy-5(e61) unc-13(e4501) I; sDP2 (1;f)* [6], DR412: *daf-15(m81)/ unc-24(e138) IV [7]*, CF1038: *daf-16(mu86) I* [8], CB1370: *daf-2(e1370) III [9]*, DR1572: *daf-2(e1368) III* [10], MT12963: *ssl-1(n4077) III/eT1 III; +/eT1 V* [11], MT13172: *+/nT1 IV[qIs51]; mys-1(n4075) V/nT1 V* [11], PD8120: *smg-1(cc546ts) I* (www.addgene.org/labs/Fire/Andrew/Vec97.pdf), KX15: *ife-2(ok306) X* [12] was backcrossed twice against wild-type N2 for lifespan analysis, DR108: *dpy-11(e224) unc-42(e270)* V, TJ356: *zIs356[daf-16::daf-16-gfp; rol-6]* IV [13], JK560: *fog-1(q253)* I [14], *uri-1(tm939)* I [15], LB127: *atp-2(ua2)* III; sDp3(III;f) [16], RW10204: *unc-119(ed3)* III; WRM0617dE06 (*pha-4::mCherry; C. briggsae unc-119 (+)*); modENCODE).

pha-4(ts): We previously generated a temperature-sensitive configuration of *pha-4* (*pha-4(ts)*) by combining *pha-4(zu225)* with *smg-1(cc546ts)* [17]. *pha-4(zu225)* contains a premature stop codon that renders *pha-4* mRNA subject to degradation by the nonsense-mediated decay (NMD) pathway [3]. *smg-1(cc546ts)* is a temperature-sensitive allele of the NMD component *smg-1* (www.addgene.org/labs/Fire/Andrew/Vec97.pdf). At 24° animals are viable whereas at 15° or 20°, animals die with reduced PHA-4 levels [3].

DNA constructs

The dsRNA plasmid for *let-363* RNAi (bSEM 911) consists of a 484 bp *let-363* fragment cloned from wild-type (N2) genomic DNA using the following primers (attB sites underlined, gene sequence bold): let-363/TOR GW-RNAi F: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT**TATCGATCAGAAACGAGCCG**-3', let-

363/TOR GW-RNAi R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT**GTAATGCATCAATTCCGGCG**-3'. The *daf-15* RNAi plasmid (bSEM 912) consists of a 449 bp *daf-15* fragment cloned from Okkema embryonic cDNA library [18] using the following primers (attB sites underlined, gene sequence bold): daf-15/RAPTOR GW-RNAi F: 5'-∑GGGGACAAGTTTGTACAAAAAAGCAGGCT**TGTGGCATGCTCAGAGAAA**-3', daf-15/RAPTOR GW-RNAi R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT**CCGTGTCCGTTGTAGTGAAA**-3'. The PCR products were inserted into pDONORdT7 [19] using a BP reaction (Invitrogen) to create RNAi entry clones, according to the manufacturers instructions. The clones were transformed into *E. coli* strain HT115 [20].

DAF-16 localization

daf-16::daf-16-gfp [13] L4 larvae, were transferred to *rsks-1(RNAi), ife-2(RNAi), pha-4(RNAi), ruvb-1(RNAi), let-363(RNAi); daf-15(RNAi)*, vector or OP50 control plates. Worms were incubated at 25°C for 48 hours on food or starved for 24 hours and imaged at the same exposure using a Zeiss Stemi SV11 Apo Microscope.

Brood Size Analysis

Wild-type L4 larvae were transferred to individual plates with 1mM IPTG and appropriate bacteria to initiate *pha-4(RNAi)* vs. L4440 vector control. Worms were incubated at 25°C and moved daily. Progeny were counted each day until reproduction ceased (2 independent experiments, $n \geq 17$ total for each RNAi treatment, average brood size ± standard deviation is reported).

Comparison of Insulin, CeTOR and *ruvb-1* phenotypes

All worms, except *daf-2*, were grown at 20°C and L3 larvae were observed under the light microscope for phenotypes. Wild-type animals were subjected to *pha-4(RNAi)* from L1 until L3 stage. *daf-2(e1370)* worms were grown at 15°C and shifted to the non-permissive temperature of 25°C at the L1 stage and analyzed at L3 for fat analysis. Resulting Dauer larvae were scored for phenotypes.

Nile Red Stains

Nile Red staining was performed according to [21]. Nile Red powder (N-1142 Molecular Probes) was dissolved in acetone at 500 mg/ml, diluted in 1X phosphate buffered saline (PBS) and added to plates already seeded with bacteria to a final concentration of 0.05

mg/ml. Starved L1 larvae (*daf-2*) were shifted to food at the non-permissive temperature of 25°C or progeny of L4 hermaphrodites (wild-type, *let-363(h111)/ dpy-5(e61)* [6] , *unc-42(e279) ruvb-1(px34)/ evl-1(ar115)* [2], *daf-15(m81)/ unc-24(e138* [7] and *daf-16(mu86)* [8]) at 20°C were imaged after 30-35 hours growth, at the L3 stage. Fluorescent images were obtained with same exposure using a Zeiss Axioskop Microscope. Black and white images were inverted for better viewing.

^{35}S-methionine incorporation

^{35}S incorporation was performed according to [22]. A temperature-sensitive allele of *fog-1(q253ts)* was used to test total protein synthesis rates in adults only. *fog-1* embryos were shifted to 25°C to cause transformation of XX animals into self-sterile females [14]. We measured the levels of radioactive methionine incorporated into protein during a 5 hr period. ^{35}S-methionine incorporation was performed as described [22] with the following changes. OP50 was cultured in LB (1ml per worm sample) containing 10µCi per ml of ^{35}S-methionine for 12 hrs. Fog-1 animals were bleached for embryos and shifted to non-permissive temperature at 25°C to cause sterility. RNAi treatments were initiated at L4 (day 0 of adulthood). L4 larva were fed *EV(RNAi), fib-1(RNAi), ruvb-1(RNAi), ife-2(RNAi)* or a combination of *let-363(RNAi); daf-15(RNAi).* Day 2 adults (100 to 200ul pellet) were mixed with radioactive bacteria and incubated at room temperature for 5 hours with shaking. At the end of this period, negative controls were produced by mixing worm samples with radioactive bacteria for 2 min. All worms were washed twice with S-Basal buffer and incubated with non-radioactive OP50 for 30 min. at room temperature with shaking to purge undigested ^{35}S-methionine labeled OP50 out of the intestine. Worms were washed twice with S-Basal buffer and flash frozen in liquid nitrogen. Frozen samples were boiled in 1% SDS, centrifuged at 14000 rpm and supernatant was removed. Protein concentrations were measured using CB-X Protein Assay. ^{35}S activity was measured by liquid scintillation using a Beckman LS 6500. The relative ^{35}S-methionine incorporation was determined by normalizing the ^{35}S-methionine incorporation levels of various RNAi animals to the ^{35}S-methionine incorporation levels of control (EV(RNAi)) animals, which was set to 100.

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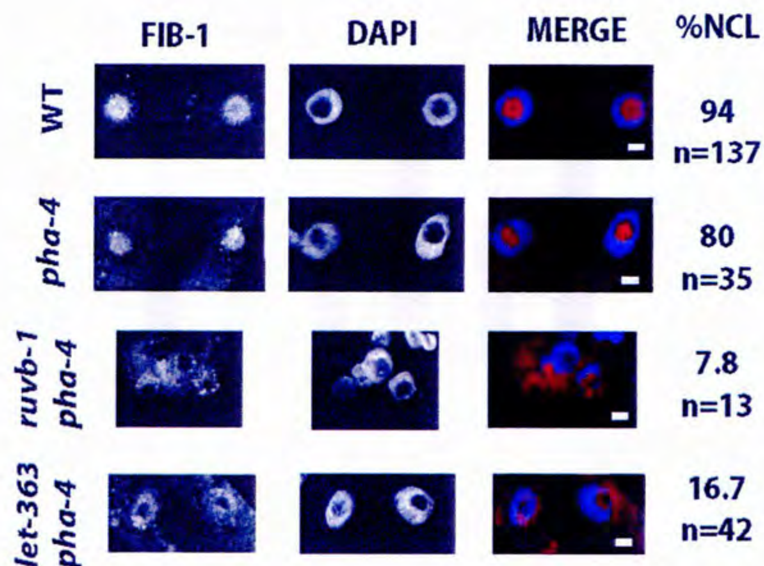
Figure S1. ***pha-4*** **is not required for mis-localization of the box C/D snoRNP complex**



Wild-type, *ruvb-1(px34)* or *let-363(h111)/TOR* larvae were stained for FIB-1 (pink) and DNA (DAPI, blue) at the L3 stage (scale bar=2 µm). *pha-4(RNAi)* was initiated at the L1 stage and analyzed at the L3 stage. Data were quantified for percent nucleolar (%NCL) and number of nuclei (n). Images were acquired using an Olympus FluoView™ FV1000 confocal microscope.

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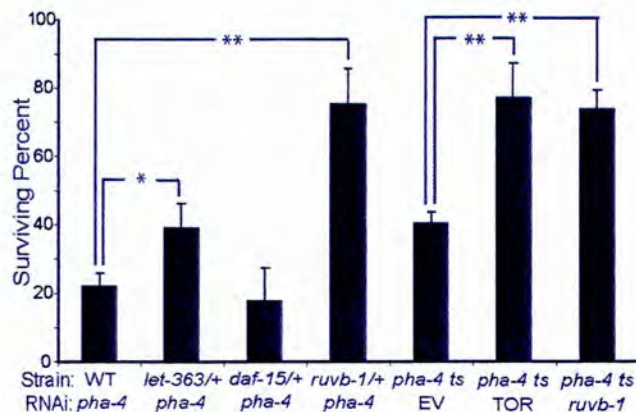
Figure S2. Genetic Interactions between *pha-4* and *ruvb-1* or CeTOR.



Suppression of the lethality associated with reduced *pha-4* by CeTOR or *ruvb-1* was performed according to [2]. Heterozygous animals *for ruvb-1(px34), let-363(h111) or daf-15(m81)* were subjected to *pha-4(RNAi)*; *GFP(RNAi)* at 1:4 dilution. Alternatively, *pha-4(ts)* animals were grown at 24°C, shifted to 20°C at the L4 stage and subjected to *GFP(RNAi)*, *ruvb-1(RNAi)* or *let-363(RNAi); daf-15(RNAi)*. Progeny were scored for survival beyond the L1 stage (25°, 2 experiments, n≥800 animals for each strain; 20°, 2 experiments, n≥250 animals total for each RNAi treatment, error bars denote standard error, **$p<0.02$, *$p=0.067$). EV, empty vector.

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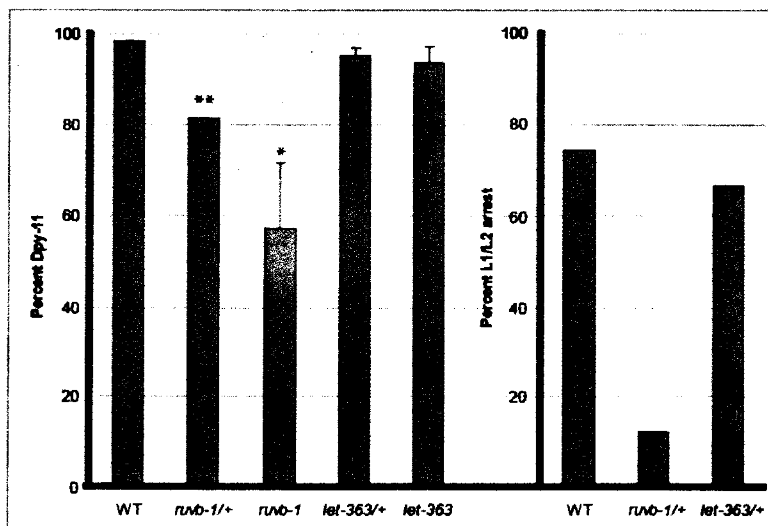
Figure S3. *ruvb-1* activity is required for efficient RNAi



***ruvb-1* but not TOR is required for RNA interference**

C. elegans ruvb-1 and its interacting partner *ruvb-2* were previously found in a screen for genes necessary for RNAi [27]. To examine the role of *ruvb-1* in RNAi, we monitored the phenotypes induced by *dpy-11(RNAi)* and *nhr-23(RNAi)* in wild-type, *ruvb-1/+* or *let-363/+* worms. WT, *unc-42(e279) ruvb-1(px34) / evl-1(ar115) V* or *let-363(h111) / dpy-5(e61)* L4 hermaphrodites were fed bacteria expressing *dpy-11* or *nhr-23* double-stranded RNA (dsRNA) at 20°C and allowed to lay eggs for 24 hours. L3 progeny were scored 2-3 days after egg-laying for Dpy-11 phenotypes (medium dumpy) (average of three experiments, n≥100 for each strain, error bars denote standard error, *p=0.021 compared to WT, **p=0.081 compared to WT, P-values determined by t-test). Progeny were scored 2 days after egg-laying for *nhr-23* (L1/L2 larval arrest) (one experiment, n≥24 for each strain) [28]. This analysis revealed that fewer *ruvb-1/+* heterozygotes had the expected phenotypes (Dumpy, Lethal) compared to wild-type or *let-363/+* animals. Next, we compared *dpy-11(e224) ruvb-1* double mutants to *dpy-11(RNAi) ruvb-1* animals to determine if the weaker phenotype reflected RNAi or some other cause. *dpy-11(e224); ruvb-1* mutants resemble *dpy-11(e224)* or *dpy-11(RNAi)*. These data confirm a role for *ruvb-1* in the effectiveness of RNAi, although not an absolute requirement. This result extends recent observations linking RNAi machinery to rRNA maturation [29]. Importantly for this study, the interaction between *pha-4* and *ruvb-1* does not rely on RNAi effects since *ruvb-1(px34)/+; pha-4(ts)* worms are viable [2].

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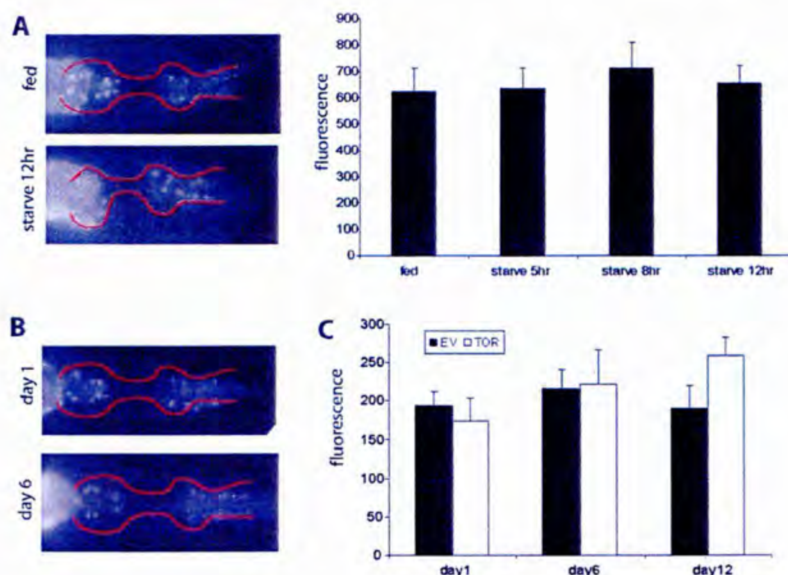
Figure S4. PHA-4 levels and localization do not change with starvation, aging or CeTOR inactivation.



PHA-4 levels and localization do not change in starvation or aging. A *pha-4::mCherry* translational fusion (RW10204; modENCODE) was used to visualize *pha-4* expression in the adult pharynx (outlined in red). A) Starvation does not alter PHA-4 protein levels or localization. Day one adult worms were washed and grown on plates with or without food at 25°C for the indicated times. Pictures of the adult pharynx were imaged at the same exposure using a Zeiss Axioskop Microscope. A threshold for fluorescence was set for each experiment and images were measured for intensity over the set threshold (error bars denote standard deviation, n≥5 worms for each condition). B) Aging does not alter PHA-4 protein levels or localization. L4 larvae (day 0) were grown at 25°C and imaged at days one and six. Pictures of the adult pharynx were imaged at the same exposure using a Zeiss Axioskop Microscope. C) Reduced CeTOR does not alter PHA-4 protein levels or localization. L4 larvae (day 0) were grown on vector (EV) or *let-363; daf-15(RNAi)* (TOR) at 25°C and imaged at days one, six or twelve. Pictures of the adult pharynx were imaged at the same exposure using a Zeiss Axioskop Microscope. A threshold for fluorescence was set for each experiment and images were measured for intensity over the set threshold (error bars denote standard deviation, n≥5 worms for each condition). Note that different thresholds were used in A) and C) so fluorescence levels cannot be compared for starvation vs. CeTOR inactivation.

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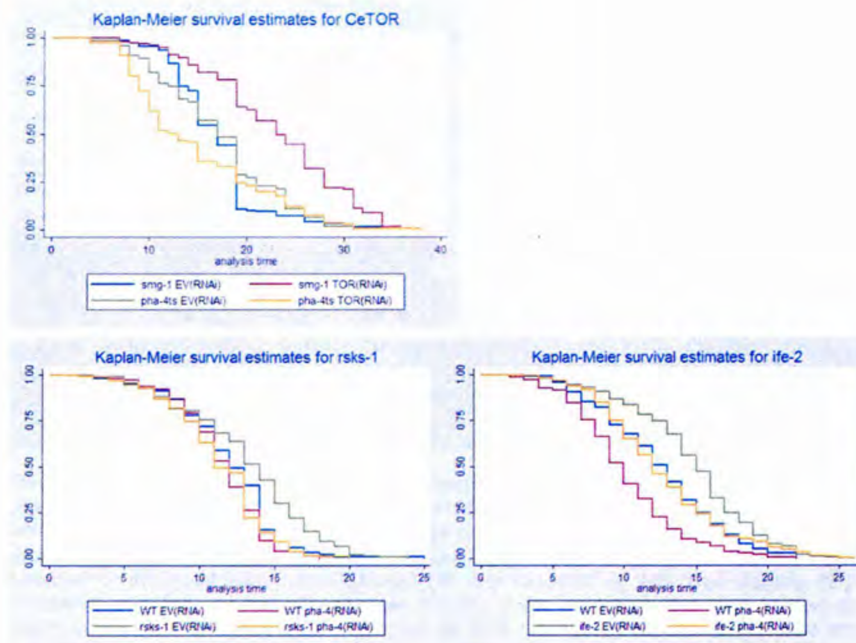
Figure S5. Kaplan-Meier Curves



Results of the multivariate Cox proportional hazards model for CeTOR, *rsks-1* and *ife-2* generated Kaplan-Meier survival estimate curves [30]. These modelling curves resemble the actual lifespan graphs presented in Figure 5A,C and D, as expected. Kaplan-Meier survival estimate curves were generated from data sets using Stata software. See Table S4 and S5 for lifespan analysis.

Table S1. *ruvb-1* **mutants have CeTOR-like phenotypes.**

Strain	L3 Arrest (%)	High Lipid (%)	Granules (%)	Alae (%)	Closed GI tract (%)	Pumping Pharynx (%)	Nucleolar Size (%)
WT n=20	0	0	0	0	0	100	23±6 (n=66)
pha-4 n=19	0	0	0	0	0	100	27±8 (n=27)
daf-2 n=19	100	100	95	100	100	0	17±4 (n=7)
let-363 n=41	98	87	98	0	0	100	13±4 (n=27)
daf-15 n=21	100	100	100	0	0	100	14±3 (n=22)
ruvb-1 n=20	100	94	100	0	0	100	17±4 (n=31)

Phenotypes of *ruvb-1(px34)* mutant were compared with those of the Insulin pathway (*daf-2(e1370ts)*), the CeTOR pathway (*let-363(h111)* or *daf-15(m81)*), or *pha-4(RNAi)*. *pha-4(RNAi)* treatment was performed on first stage (L1) larva and scored at L3 or adult. Larval arrest was determined to be at the L3 stage by body size, arrested gonad extension and arrested or perturbed vulval development. Fat was detected by Nile Red staining [21]. Epidermal granules are refractile storage vesicles in the epidermis and were scored for increased abundance compared to wild type by light microscopy (Granules) [6]. Alae are cuticular structures of the epidermis that are specific to Dauer larva and adults [7]. A closed digestive tract was scored by observation of a closed mouth, thin non-pumping pharynx and thin intestinal lumen under the light microscope [7]. Nucleolar size was scored by measurement of total nuclear area divided by nucleolar area in epidermal cells. Nucleolar size is shown in percent of total nuclear area with standard deviation. P-values were determined by t-test (all compared to WT; *pha-4(RNAi)* $p=0.01$; *daf-2* $p=0.01$; *let-363* $p<0.0001$; *daf-15* $p<0.0001$; *ruvb-1* $p<0.0001$). *Strain n* is number of animals scored; *Nucleolar n* is number of nuclei scored.

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Table S2. Phenotypes associated with Box C/D snoRNP components resemble those associated with CeTOR

Function	Gene	Homology	Larval Arrest	High Lipid	Granules	Nucleolus area (%)
--	WT	--	-	-	-	23±6 (n=66)
Fox TF	*pha-4*[a]	FOXA	-	-	-	27±8 (n=27)
TOR	*let-363*[abc]	TOR kinase	++	++	++	13±4 (n=27)
TOR	*daf-15*[abc]	Raptor	++	++	++	14±3 (n=22)
TOR	*ruvb-1*[ab]	helicase	++	++	++	17±4 (n=31)
TIP60	*mys-1*[abc]	TIP60	-	-	-	ND
SWR1	*ssl-1*[abc]	Swr-1	-	-	-	ND
DNA repair	ZK1127.4[c]	BRCA2	-	ND	ND	ND
DNA repair	*brc-2*[c]	BRCA2	-	ND	ND	ND
PRC-2	*mes-2*[c]	Methyl-transferase	-	ND	ND	ND
prefoldin	*uri-1*[bc]	URI	++	+	+	23±5 (n=27)
Box C/D snoRNP	*nol-5*[ac]	Nop58	++	-	++	16±7 (n=8)
Box C/D snoRNP	K07C5.4[a]	Nop56	++	-	++	17±2 (n=4)
Box C/D snoRNP	*fib-1*[a]	Fibrillarin	++	-	++	13±4 (n=10)
ATP synthase	*atp-2*[bc]	β-subunit	++	ND	-	45±7 (n=6)

We examined *C. elegans* genes homologous to components of RUVB-containing complexes in other organisms for CeTOR-like phenotypes: MYS-1 (VC5.4) and SSL-1 (Y111B2A.22) are homologous to proteins in the transcriptional complex ESA1/SWR1 [11, 23]. DNA repair was examined with *C. elegans* BRCA2 (ZK1127.4) (WormBase) and *brc-2* (T07E3.5) [24]. There is no obvious orthologous complex to PRC2 in *C. elegans* so we examined *mes-2*, which is orthologous to the Enhancer of Zeste histone methyltransferase required to target PRC2 in other animals (R06A4.7) [25]. *C. elegans uri-1* was used to examine the URI complex (C55B7.5) [15]. The Box C/D snoRNP complex was predicted to contain core components *nol-5* (Nop58) (W01B11.3), K07C5.4 (Nop56), and *fib-1* (fibrillarin) (T01C3.7) [26]. We also examined another gene that causes L3 larval arrest: *atp-2, which* is orthologous to the β-subunit of ATP synthase. WormBase website, WS180. www.wormbase.org.

Phenotypes were determined by [a] RNAi, [b] mutants or [c] literature. Larval arrest was determined to be in the ~L3 larval stage by observation of body size, arrested gonad development and lack of vulval development. Fat was detected by Nile Red staining [21]. Epidermal granules were scored for increased abundance compared to wild type under the light microscope. Nucleolar size was scored by measurement of total nuclear area divided by nucleolar area in epidermal cells. Nucleolar size is shown in percent of total nuclear area

with standard deviation. P-values were determined by t-test (all compared to WT; *pha-4(RNAi)* p=0.01; *let-363* p<0.0001; *daf-15* p<0.0001; *ruvb-1* p<0.0001; *uri-1* p=0.75; *nol-5* p=0.005; KO7C5.4 p=0.04; *fib-1* p<0.0001; *atp-2* p<0.0001). Nucleolar n is number of nuclei scored. (++ = >50% showed phenotype, + = <50% showed phenotype, - = 0% showed phenotype, ND = not determined).

Table S3. Lifespan Analysis for *ruvb-1*

Strain/RNAi	Temp (°C)	Mean Lifespan (days)	75% Lethal	No. of animals	%change in lifespan vs. WT	P-value
WT/EV	25	10.7	14	110/140 (8)		
WT/*ruvb-1*	25	11.5	14	105/138 (11)	+7.5	0.474
WT/EV	25	11.6	14	84/92 (2)		
WT/*ruvb-1*	25	12.2	16	62/103 (3)	+5.2	0.34

Lifespan analysis was performed as described previously [22] with the following modifications. Mean adult lifespan, in days, of wild-type animals with reduced *ruvb-1* induced by RNAi was measured, beginning at the L4 stage. Bacteria bearing empty vector served as a control. 75% lethality reflects the mean lifespan, in days, of the 75th percentile (the age at which the fraction of animals alive reaches 0.25). Number (No.) of animals reflects the number of observed deaths/total number of animals subjected to RNAi treatment. The difference between these numbers represents the number of animals censored during the experiment (number of ruptured animals are shown in parentheses), and includes animals that ruptured, crawled off the plate or exhibited progeny hatching internally. *P* values were calculated by pair-wise comparisons to the control of the experiment by using the Log-rank test using Stata software.

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Table S4. Lifespan Analysis for CeTOR, *daf-2* and *pha-4*

Strain/RNAi	Temp (°C)	Mean Lifespan (days)	75% Lethal	No. of animals	%change in lifespan vs. § strain	P-value	%change in lifespan vs. ‡ strain	P-value
WT (log rank)								
§ WT/EV	25	11.8	14	130/149(7)				
WT/*pha-4*	25	10.2	12	124/143(8)	-13.6	<0.0001		
§ WT/EV	25	11	13	125/137(4)				
WT/*pha-4*	25	10.6	13	130/149(5)	-3.6	0.563		
§ *smg-1*/EV	15	22.6	29	60/115(10)				
pha-4ts/EV	15	15.8	21	76/89(0)	-30.1	<0.0001		
§ *smg-1*/EV	15	23.5	28	38/94(53)				
pha-4ts/EV	15	21.5	25	100/103(2)	-8.5	0.0826		
DAF-2 (log rank)								
§ *daf-2*/EV	25	25.9	31	61/63(0)				
daf-2/*pha-4*	25	24.4	30	65/65(0)	-5.8	0.136		
§ WT/EV*	25	11.6	14	84/92(2)				
‡ *daf-2*/EV*	25	23.6	28	131/137(0)	+103.4	<0.0001		
daf-2/*pha-4**	25	25.2	30	147/149(0)	+117.2	<0.0001	+6.8	0.1168
TOR/RAPTOR (log rank)								
§ *smg-1*/EV	15	17.8	21	96/131(19)				
smg-1/TOR	15	23.6	29	94/123(11)	+32.6	<0.0001		
EXP1								
§ *smg-1*/EV*	15	18.1	19	65/77(5)				
pha-4ts/EV*	15	13.8	19	37/42(1)	-23.8	0.0017		
‡ *smg-1*/TOR*	15	21.3	26	64/76(2)	+17.7	0.0077		
pha-4ts/TOR*	15	12.2	18	51/60(1)	-32.6	<0.0001	-42.7	<0.0001
EXP2								
§ *smg-1*/EV	15	15.8	17	75/87(8)				
pha-4ts/EV	15	17.9	21	95/98(0)	+13.3	0.00375		
‡ *smg-1*/TOR	15	23.6	28	77/87(2)	+33.1	<0.0001		
pha-4ts/TOR	15	15.98	21	90/93(0)	+1.1	0.605	-32.3	<0.0001
§ *smg-1*/EV	15	18.3	23	42/212(170)				
pha-4ts/EV	15	19	23	122/131(13)	+3.8	0.609		
‡ *smg-1*/TOR	15	20.2	23	45/106(61)	+10.4	0.206		
pha-4ts/TOR	15	18.85	23	84/98(14)	+3	0.964	-6.7	0.2957

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combined TOR/RAPTOR (Cox regression)

15°C TOTAL (EXP1 + EXP2)								
§ *smg-1*/EV	15	17	19	140/164(13)				
pha-4ts/EV	15	17.2	21	132/140(1)	+1.2	0.4067		
‡ *smg-1*/TOR	15	23.1	28	141/163(4)	+35.9	<0.0001		
pha-4ts/TOR	15	14.9	19	141/153(1)	-12.4	0.0428	-35.5	<0.0001
***pha-4ts*/EV vs. *pha-4ts*/TOR							<0.0001	

Lifespan analysis was performed as described previously [22] with the following changes. Mean adult lifespan, in days, of *daf-2(e1368) [10], pha-4(zu225);smg-1(cc546ts)*(called *pha-4ts) [3], smg-1(cc546ts)* mutants (www.addgene.org/labs/Fire/Andrew/Vec97.pdf) or wild-type animals with reduced *pha-4* or CeTOR signaling (combined *let-363* and *daf-15)* induced by RNAi, beginning at the L4 stage. Bacteria bearing empty vector (EV) L4440 served as a wild-type (WT) control. 75% lethality reflects the mean lifespan, in days, of the 75th percentile (the age at which the fraction of animals alive reaches 0.25). Number of animals (No.) reflects the number of observed deaths/total number of animals subjected to RNAi treatment. The difference between these numbers represents the number of animals censored during the experiment (number of ruptured animals are shown in parentheses), and includes animals that ruptured, crawled off the plate or exhibited progeny hatching internally. P-values were calculated using the log-rank and multivariate Cox proportional hazards model using Stata Software. '*', data shown in Fig. 5. **Log rank tests were used in pair wise comparisons of wildtype, mutant, treatment, and non-treated groups. These results were compared to a multivariate Cox proportional hazards model which, in addition to simultaneously looking at mutation and treatment effects, could include information from the censored subjects. The model was used to determine the effects of mutation and treatment including their interaction. The interaction term indicates whether the treatment effect was different in wildtype versus mutant.

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Table S5. Lifespan Analysis for *rsks-1* and *ife-2*

Strain/RNAi	Temp (°C)	Mean Lifespan (days)	75% Lethal	No. of animals	%change in lifespan vs. § strain	P-value	%change in lifespan vs. ‡ strain	P-value
RSKS-1 (log rank)								
EXP1								
§ WT/EV*	25	11.96	14	85/119(18)				
WT/pha-4*	25	11.1	13	106/122(7)	-7.2	0.0075		
‡ rsks-1/EV*	25	12.98	15	91/101(3)	+8.5	0.0011		
rsks-1/pha-4*	25	10.4	13	93/97(0)	-13	0.0001	-19.9	<0.0001
EXP2								
§ WT/EV	25	11.5	14	84/106(22)				
WT/pha-4	25	11.8	14	95/105(10)	+2.6	0.9239		
‡ rsks-1/EV	25	13.3	17	69/90(21)	+15.7	0.0023		
rsks-1/pha-4	25	12.2	14	89/99(10)	+6.1	0.3002	-8.3	0.0040
§ WT/EV	20	16.6	19	36/44(5)				
WT/pha-4	20	13.8	16	37/41(1)	-16.9	0.0004		
‡ rsks-1/EV	20	19.5	24	55/75(11)	+17.5	0.0006		
rsks-1/pha-4	20	14.7	17	59/63(3)	-11.4	0.00385	-24.6	<0.0001
§ WT/EV	20	16.7	20	96/119(7)				
WT/pha-4	20	13.9	16	83/114(16)	-16.8	<0.0001		
‡ rsks-1/EV	20	19	26	75/116(24)	+13.8	<0.0001		
rsks-1/pha-4	20	16.4	20	99/122(2)	-1.8	0.7952	-13.7	<0.0001
Cumulative RSKS-1 (Cox regression)								
25°C EXPS TOTAL								
§ WT/EV	25	11.98	14	169/225(40)				
WT/pha-4	25	11.55	14	229/227(17)	-3.6	0.0285		
‡ rsks-1/EV	25	13.25	16	160/191(24)	+10.6	<0.0001		
rsks-1/pha-4	25	11.41	13	182/196(10)	-4.75	0.0852	-13.9	<0.0001
**WT/pha-4 vs. rsks-1/pha-4								0.007
IFE-2 (log rank)								
EXP1								
§ WT/EV*	25	11.16	14	77/91 (5)				
WT/pha-4*	25	9.5	11	80/100 (7)	-14.9	0.0032		
‡ ife-2/EV*	25	13.6	16	101/118 (0)	+21.9	<0.0001		
ife-2/pha-4*	25	12.15	14	109/117 (4)	+8.9	0.0664	-12.3	0.0023
EXP2								
§ WT/EV	25	13.3	17	74/105(30)				
WT/pha-4	25	10.2	13	85/100(15)	-23.3	<0.0001		
‡ ife-2/EV	25	15.5	19	89/106(17)	+16.5	0.0023		
ife-2/pha-4	25	13.1	17	76/99(23)	-1.5	0.663	-15.5	0.0656
§ WT/EV	25	10.7	14	110/140 (8)				
‡ ife-2/EV	25	13.8	17	108/127 (21)	+28.97	<0.0001		
ife-2/pha-4	25	12.1	15	102/127 (24)	+13.1	0.0156	-12.3	0.0001

§ *ife-2*/EV	25	16.8	19	135/145 (10)				
ife-2/pha-4	25	13.1	16	131/140 (9)	-22	<0.0001		
§ *ife-2*/EV	20	16.9	20	101/149 (15)				
ife-2/pha-4	20	14.3	15	93/150 (38)	-15.4	<0.0001		
Cumulative IFE-2 (Cox regression)								
25°C EXPS TOTAL (EXP1 + EXP2)								
§ WT/EV	25	12.5	15	151/196(35)				
WT/*pha-4*	25	10.1	12	165/200(22)	-19.2	<0.0001		
‡ *ife-2*/EV	25	14.7	17	190/224(17)	+17.6	<0.0001		
Ife-2/pha-4	25	12.7	15	185/216(27)	+1.6	0.6514	-13.6	0.0001
**WT/*pha-4* vs. *ife-2/pha-4*								0.158

Lifespan analysis was performed as described previously [22] with the following changes. Mean adult lifespan, in days, of *ife-2(ok306) [12], rsks-1(ok1255) [4]* mutants or wild-type animals with reduced *pha-4* induced by RNAi beginning at the L4 stage. Bacteria bearing empty (EV) vector served as a wild-type (WT) control L4440. 75% lethality reflects the mean lifespan, in days, of the 75th percentile (the age at which the fraction of animals alive reaches 0.25). Number of animals reflects the number of observed deaths/total number of animals subjected to RNAi treatment. The difference between these numbers represents the number of animals censored during the experiment (number of ruptured animals are shown in parentheses), and includes animals that ruptured, crawled off the plate or exhibited progeny hatching internally. P-values were calculated using the log-rank and multivariate Cox proportional hazards model using Stata Software. '*', data shown in Fig. 5. **Log rank tests were used in pair wise comparisons of wildtype, mutant, treatment, and non-treated groups. These results were compared to a multivariate Cox proportional hazards model which, in addition to simultaneously looking at mutation and treatment effects, could include information from the censored subjects. The model was used to determine the effects of mutation and treatment including their interaction. The interaction term indicates whether the treatment effect was different in wildtype versus mutant.

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Table S6. Genetic Interactions between *pha-4* and *ife-2* or *rsks-1*.

Strain	RNAi mixture	%alive (±stdev)	Total animals
WT	*pha-4*	6.8 ±0.2	270
ife-2	*pha-4*	3.7±0.4	408
WT	*pha-4:gfp 1:4*	62.7±0.1	273
ife-2	*pha-4:gfp 1:4*	11.8±0.03	278
WT	*pha-4:gfp 1:10*	97.4±0.01	281
ife-2	*pha-4:gfp 1:10*	62.3±0.1	422
WT	*pha-4:gfp 1:4*	2.3 ±0.1	784
rsks-1	*pha-4:gfp 1:4*	6.8±0.06	759
WT	*pha-4:gfp 1:4*	3.5±0.04	204
rsks-1	*pha-4:gfp 1:4*	18.8±0.06	358
WT	*pha-4:gfp 1:8*	5.5±0.04	1248
rsks-1	*pha-4:gfp 1:8*	19.2±0.04	1446

Suppression of the lethality associated with reduced *pha-4* by CeTOR or *ruvb-1* was performed according to [2]. Different ratios of *pha-4(RNAi)* (bSEM 865) to *GFP(RNAi)* [20] were used to partially inactivate PHA-4. Wildtype animals and animals mutant *for ife-2(ok306)[12] or rsks-1(ok1255)[4]* were subjected to *pha-4(RNAi)*; *GFP(RNAi)*. Progeny were scored for survival beyond the L1 stage.

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

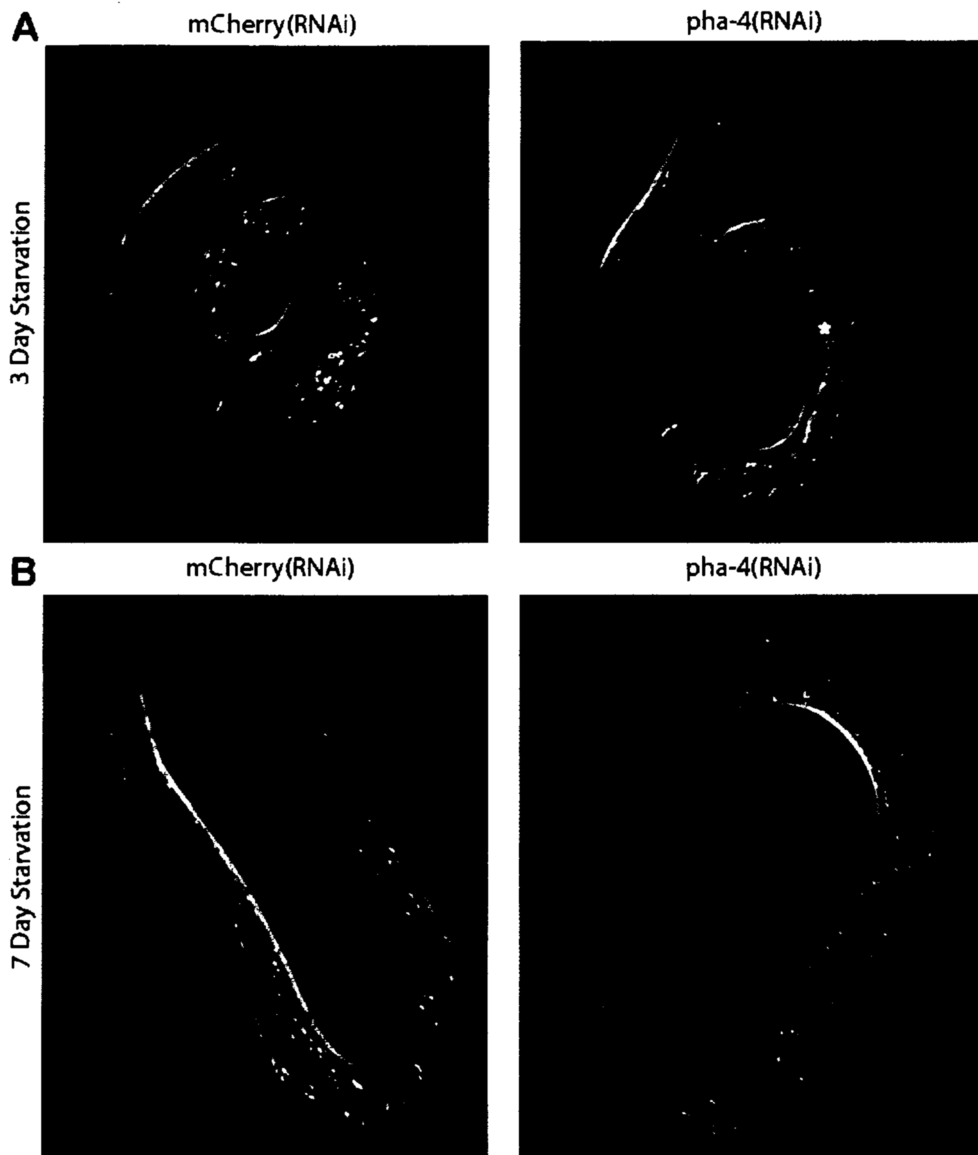


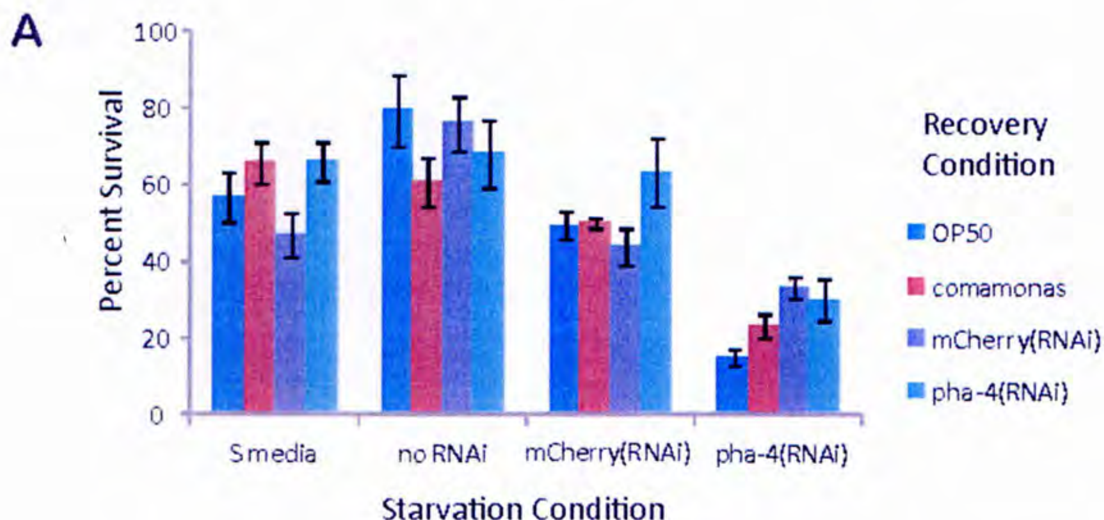
Figure B.1. Soaking *pha-4(RNAi)* does not cause morphological defects in the pharynx.

A-B. L1 larvae were exposed to *mCherry(RNAi)* or *pha-4(RNAi)* without food for 3-7 days. Using previously published morphological defects due to loss of *pha-4* as a guide, worms were checked for defects using DIC. Pharynx is highlighted in red, *intestinal vacuoles. A total of 34-39 L1s were visualized for each RNAi treatment.

A. L1 larvae starved for 3 days. There is no difference in pharynx morphology and representative images are shown above. 38.5% of worms treated with *pha-4(RNAi)* show intestinal vacuoles. These vacuoles are not seen in *mCherry(RNAi)* treated worms.

B. L1 larvae starved for 7 days. There is no difference in pharynx morphology and representative images are shown above.

Figure B.2. *pha-4* may be required for feeding recovery after starvation.
A. Loss of *pha-4* causes no defects in recovery on different kinds of bacteria. L1 larvae were starved in S media, no RNAi, *mCherry(RNAi)* or *pha-4(RNAi)*. Number of worms past L1 were counted after 2 days. Results reflect a representative experiment, n=300-500 worms counted for each condition per experiment, error bars represent standard error.
B. Loss of *pha-4* causes delayed feeding recovery after 7 days of starvation. Wild-type worms were treated with *mCherry(RNAi)* or *pha-4(RNAi)* without food for 3 or 7 days. To recover from starvation, worms were placed onto OP50 bacteria mixed with 0.5 μm fluorescent beads for 2-6 hours or 10-12 hours. Worms were mounted on slides and scored for beads in the intestine. Percentage reflects number of worms with beads in the intestine. Representative images are shown for each condition and beads are colored in red. Results represent one experiment, n=13-70 worms counted for each condition per timepoint.



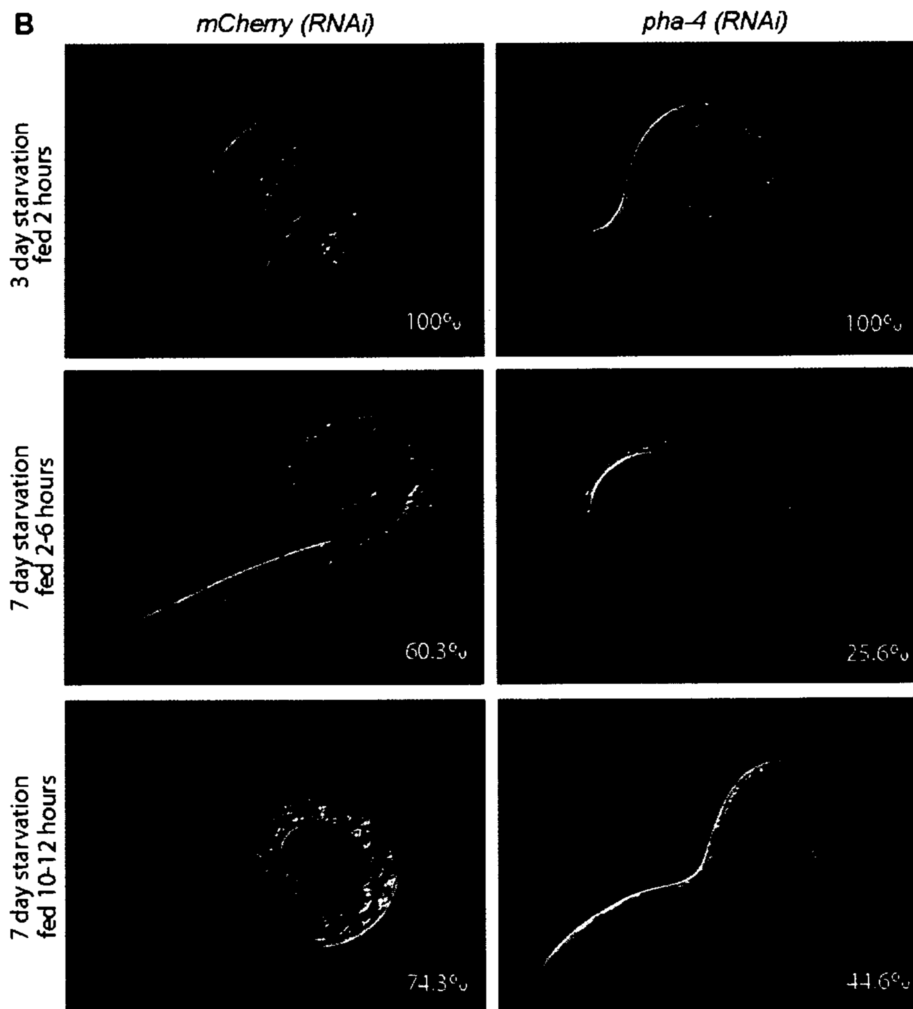
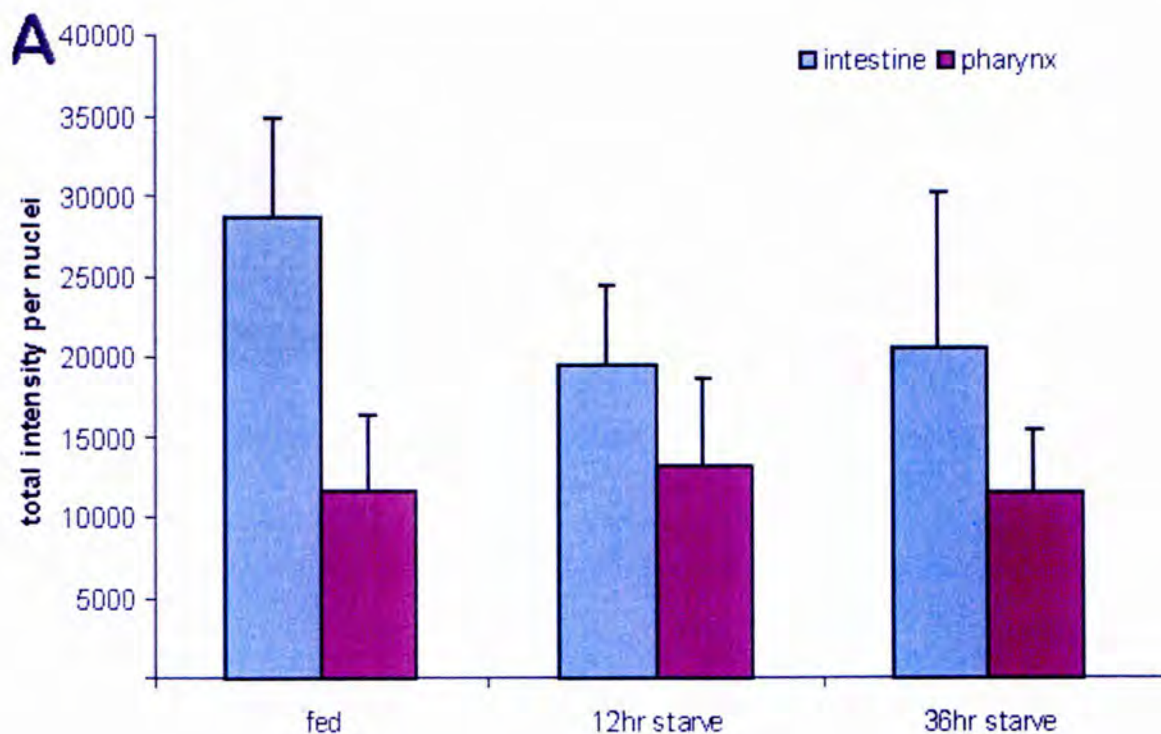
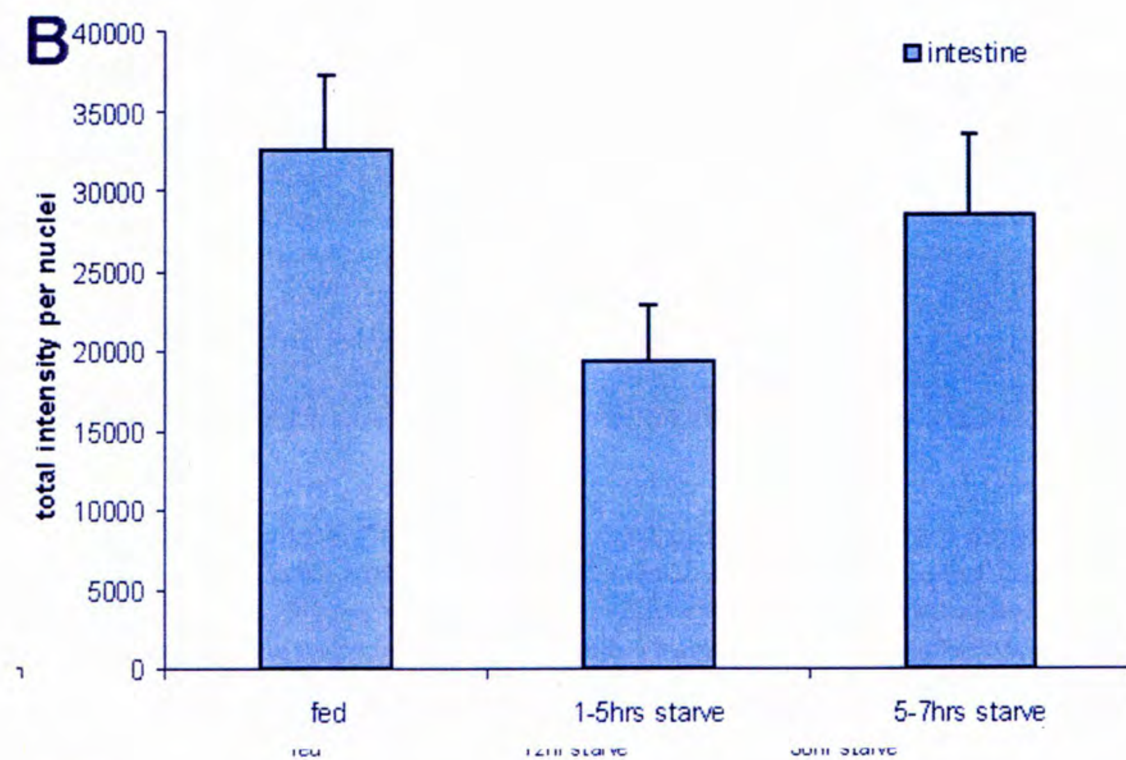
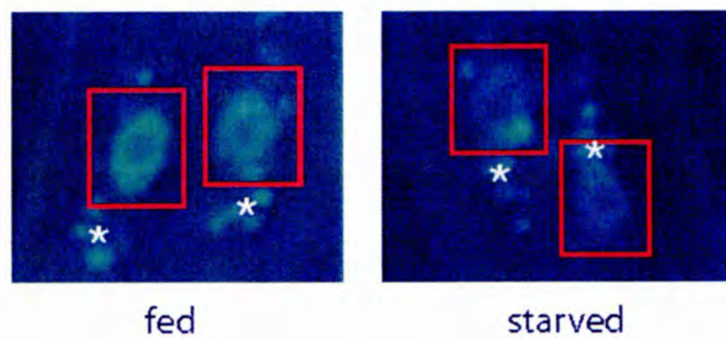

B
mCherry (RNAi)
pha-4 (RNAi)
3 day starvation fed 2 hours
7 day starvation fed 2-6 hours
7 day starvation fed 10-12 hours
100%
100%
60.3%
25.6%
74.3%
44.6%

Figure B.3. PHA-4 levels and localization do not change in starvation.
A. PHA-4 levels do not change during long-term starvation. PHA-4::YFP was used to visualize PHA-4 expression in L1 larvae during starvation. L1 larvae were fed or starved for 12 hours. Pictures were taken at the same exposure for pharynx and intestine. Nuclei were identified by DIC. Individual nuclei were measured for total intensity using Image J software. Results are an average of 5-10 nuclei per worm, n=15 worms, error bars represent standard deviation.
B. PHA-4 levels do not change during short-term starvation. PHA-4::YFP was used to visualize PHA-4 expression in L1 larvae during starvation. L1 larvae were fed or starved for 1-5hrs and 5-7hrs. Pictures were taken at the same exposure for intestine only. Nuclei were identified by DIC. Individual nuclei were measured for total intensity using Image J software. Results are an average of 5-10 nuclei per worm, n=12 worms, error bars represent standard deviation.
C. PHA-4 remains in the nucleus during starvation. PHA-4::YFP was used to visualize PHA-4 expression in L1 larvae intestine during starvation. L1 larvae were fed or starved for 12 hours. Pictures were taken at the same exposure. Red boxes outline intestinal nuclei, *autofluorescence due to intestinal granules.







fed starved

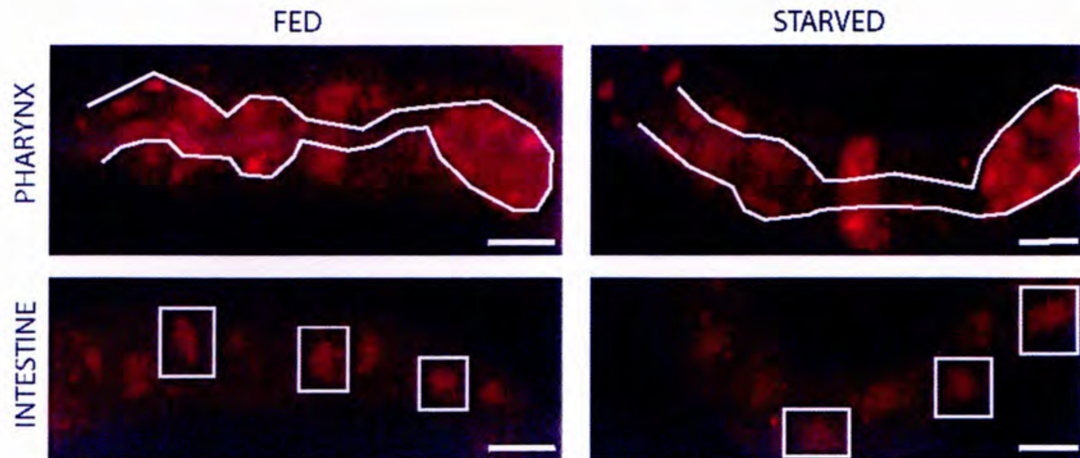


Figure B.4. Endogenous PHA-4 levels and localization are unchanged during starvation. L1 were fed or starved for 12 hours and stained with an antibody that recognizes PHA-4. We see no difference in PHA-4 levels and individual nuclei can be visualized in both the pharynx and intestine. Pharynx is outlined in white. Intestinal nuclei were identified by DAPI staining and are marked with white boxes. Scale bars are 5 μm.