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Genetic Analysis of Feeding Behavior and Fat Storage in *C. elegans*

by

Elisabeth Greer

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

to my husband

thank you

Abstract

Obesity is a disorder affecting many Understanding disorders in metabolism requires the identification of molecular and neural circuits underlying energy balance. Here we investigate the coordination between feeding behavior and fat storage in response to changing environmental conditions in *C. elegans*. The TGF- β ligand DAF-7 is expressed in two sensory neurons and is responsive to environmental conditions such as food availability and population density. Decreases in TGF- β signaling lead to increased fat storage, decreased feeding rate, and in young animals' entry into a hibernating dauer state. Here we show that DAF-7 signals to two interneurons to regulate fat storage, feeding, and dauer formation. However, within these interneurons different molecular signals independently regulate feeding, fat storage, and dauer formation. TGF- β signaling inhibits fat storage through G-protein signaling, despite increasing feeding through the neurotransmitters tyramine and octopamine. We also identify ~20 additional *C. elegans* genes that regulate feeding behavior and fat storage. Taken together, this work elucidates the complex neural and molecular circuits coordinating metabolism and behavior in nutrient limiting conditions.

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Chapter I: Introduction

The obesity epidemic

Obesity is a disease affecting much of the world's population. Studies have found that 20% of men and 25% of women in the United States are obese (Kuczmarski, Flegal et al. 1994). In developed nations, obesity contributes 2-7 % of total health care costs (Seidell 1995). Obesity is defined by the body-mass index (BMI), which is a ratio of weight to height. Obese individuals have high BMI's that correlate with increased risk for developing diabetes, heart disease, and respiratory problems (Kopelman 2000). At a basic level, obesity is a misregulation of energy intake and energy expenditure. However, obesity is affected by multiple factors such as genetics, environment, and culture. Studies predict that obesity has a heritability between 50 and 90% (Barsh, Farooqi et al. 2000). Genetics play an important role in the likelihood that an individual will be obese, yet genetic risk factors have been more difficult to identify due to the polygenic nature of the disorder (Barsh, Farooqi et al. 2000). Understanding obesity requires the identification of genes and environmental factors leading to the disorder.

Overview of pathways regulating energy homeostasis in mammals

Mammals regulate feeding behavior as well as nutrient storage in order to maintain energy homeostasis. Lesion studies first highlighted the importance of the central nervous system (CNS), specifically the hypothalamus in regulating feeding behavior and weight gain (Seeley and Woods 2003). Brain lesions could produce profound increases or decreases in food intake and weight gain depending on which particular hypothalamic region was targeted (Seeley and Woods 2003).

In mammals, feeding behavior and fat storage are impacted by four major types of signals: signals from the sites of nutrient storage, signals from the gastrointestinal tract, signals from CNS sensing of nutrients, and signals from reward pathways (Morton, Cummings et al. 2006). Together these cues impinge upon the hypothalamus to coordinate nutrient intake with energy expenditure.

Hormones released from the periphery including sites of nutrient storage were the first molecules recognized to profoundly regulate feeding behavior and energy expenditure. The hormones insulin and leptin are both satiety signals that are released in response to nutrient influx and act in the CNS to limit feeding behavior and promote energy expenditure (Flier 2004). Insulin is released from pancreatic β -cells and acts peripherally to promote uptake of glucose, while in the CNS it acts to inhibit food intake (Schwartz, Woods et al. 2000). Leptin is released from adipose tissue in proportion to the amount of fat stores present. Leptin also binds to receptors in the hypothalamus to inhibit feeding and promote energy expenditure (Seeley and Woods 2003; Morton, Cummings et al. 2006). Mutations in the leptin peptide or its receptor cause profound obesity accompanied with hyperphagia, or over-eating, and reduced energy expenditure (Zhang, Proenca et al. 1994; Tartaglia, Dembski et al. 1995).

Hormones produced in the gastrointestinal tract also regulate energy homeostasis. Examples include the secreted hormone ghrelin, which stimulates feeding, and the peptides YY₃₋₃₆ and CCK, which reduce food intake (Morton, Cummings et al. 2006). Levels of ghrelin rise before a meal and fall sharply after feeding. Ghrelin receptors are expressed in the hypothalamus and are thought to act in opposition to leptin signals on these same neurons (Inui 2001).

Insulin, leptin, and ghrelin are all hormones that are released in the periphery and that act in the CNS to regulate feeding behavior and energy expenditure. In addition to these hormonal pathways, the CNS directly monitors energy supplies through detection of metabolites. In adipose tissue fatty acid synthase (FAS) synthesizes long-chain fatty acids from malonyl-CoA. Long-chain fatty acids can be stored as fuel for the cells. Interestingly, inhibition of fatty acid synthase in the CNS reduces feeding behavior, suggesting that hypothalamic neurons can directly sense calorie surpluses and modulate behavior accordingly (Seeley and Woods 2003; Unger 2004).

The palatability of food also affects feeding behavior through activation of reward pathways in the brain. Visual, olfactory, and taste cues all impact the perceived food reward and regulate feeding behavior (Morton, Cummings et al. 2006). Researchers have observed that both food and substance abuse lead to compulsive behaviors and adaptations in the brain (Volkow and Wise 2005). For example, there are fewer D2-type dopamine receptors in obese individuals, suggesting a decreased reward value for food intake in obese individuals (Wang, Volkow et al. 2001). Dopamine neurons in the midbrain ventral tegmental area (VTA) are central to the reward pathways underpinning drug addiction. These VTA neurons express leptin receptors and innervate the nucleus accumbens, which project to neurons of the hypothalamus (Cota, Barrera et al. 2006). Exogenous leptin applied to the VTA can reduce food intake, while loss of leptin signaling on VTA neurons increases food intake (Hommel, Trinko et al. 2006). While this demonstrates that leptin can regulate dopamine neurons, perhaps making food less palatable, it is still unclear how or if sensory information impacts the hypothalamic circuits that regulate energy expenditure and feeding behavior.

Neural circuits regulating energy balance

As discussed above, signals from the gut, energy stores, and sensory systems all converge on the hypothalamus to regulate energy expenditure and feeding behavior. Two types of neurons in the arcuate nucleus of the hypothalamus act in opposing ways to affect feeding behavior and energy storage. Pro-opiomelanocortin (POMC) neurons of the arcuate nucleus produce POMC, which is cleaved into α -MSH, which binds to melanocortin type 4 receptors (MC4R). Melanocortin signaling through MC4R reduces food intake and increases energy expenditure. Administration of α -MSH to the CNS reduces food intake while loss of MC4R results in severe obesity in mice and humans (Flier 2004). Thus, through expression of α -MSH POMC neurons inhibit feeding behavior.

The second set of neurons in the arcuate nucleus express neuropeptide Y (NPY) and agouti-related protein (AgRP) peptides, which promote feeding behavior. AgRP is a high affinity antagonist of MCR4 and acts in opposition to α -MSH (Schwartz, Woods et al. 2000). Central administration of NPY increases food intake through NPY receptors Y_1 and Y_5 (Crowley, Yeo et al. 2002). Both POMC and NPY/AgRP neurons express leptin and insulin receptors; however, these hormones affect POMC and NPY/AgRP neurons in opposing manners. Leptin increases the activity of POMC neurons in addition to increasing the expression and release of α -MSH (Cone 2005). In contrast, leptin decreases expression and release of NPY and AgRP, and inhibits the activity of these neurons (Schwartz, Woods et al. 2000; Elmquist and Flier 2004; Horvath and Diano 2004). This circuit is reinforced by inhibitory connection from NPY/AgRP neurons to POMC neurons (Cone 2005).

Focusing on the role of arcuate POMC and AgRP/ NPY neurons, recent studies have begun to functionally assess the role of these neurons in energy homeostasis and feeding. Selective knockout of leptin receptors on POMC neurons results in a modest increase in body weight; however, there is no change in food intake or energy expenditure (Balthasar, Coppari et al. 2004). Leptin expressing neurons outside the arcuate nucleus also regulate fat storage since loss of leptin receptors on neurons of the ventromedial hypothalamus (VMH) also results in weight gain (Dhillon, Zigman et al. 2006). Animals lacking leptin receptor expression on VMH neurons increase food intake and have decreased energy expenditure when placed on a high fat diet (Dhillon, Zigman et al. 2006). Functional studies show that neurons of the arcuate as well as the VMH require leptin signaling to appropriately regulate energy homeostasis (Balthasar 2006).

POMC and NPY/AgRP neurons project to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA). The importance of these nuclei in weight homeostasis was first uncovered through lesion studies. Lesions of the PVN result in hyperphagia and obesity, while lesions of the LHA decrease feeding behavior and result in weight loss. POMC and NPY/AgRP neurons innervate both the PVN and LHA areas of the brain (Schwartz, Woods et al. 2000). Since both POMC and NPY/AgRP neurons affect melanocortin signaling, researchers examined the role of MC4R on PVN neurons in regulating energy homeostasis. Reactivation of MC4R in the PVN partially rescues the increased fat storage phenotype of MC4R mutant animals (Balthasar, Dalgaard et al. 2005). Surprisingly, reactivation of MC4R in the PVN suppresses the increased food intake phenotype but not the decreased energy expenditure phenotype of MC4R mutant animals (Balthasar, Dalgaard et al. 2005). Thus, expression of MC4R in other sites must

regulate energy expenditure (Balthasar 2006). Studies testing the role of proposed neural circuits are central to understanding obesity. However, transgenic mice are difficult to produce and these studies are difficult and laborious in mouse models of obesity.

Dauer formation and fat storage in *C. elegans*

C. elegans is an emerging model organism for the study of obesity (Ashrafi, Chang et al. 2003; McKay, McKay et al. 2003). Alterations in *C. elegans* metabolism were first described in animals entering the dauer or hibernating phase. Larval animals integrate signals about population density, food availability, and temperature and decide whether to enter the dauer state or become reproductive adults (Riddle, Blumenthal et al. 1997). Unfavorable environmental conditions such as high population density, food scarcity, and high temperatures promote entry into the dauer state (Golden and Riddle 1982; Golden and Riddle 1984; Golden and Riddle 1984). *C. elegans* assess population density through a constitutively secreted dauer pheromone (Golden and Riddle 1982). As the population density increases so does the concentration of dauer pheromone, which favors dauer development (Riddle, Blumenthal et al. 1997).

Dauer animals are well equipped to survive these harsh conditions due to morphological changes in the cuticle and the presence of internal plugs to prevent dehydration (Cassada and Russell 1975; Riddle, Swanson et al. 1981). In addition, dauer animals have increased fat stores, altered metabolic rate, and do not feed (Wadsworth and Riddle 1989; Riddle and Albert 1997). Normally, harsh environmental conditions will promote dauer formation; however, mutations in pathways that regulate dauer entry will enter the dauer stage constitutively even in the presence of abundant food and low temperature (Riddle, Blumenthal et al. 1997). These dauer constitutive (*daf-c*) mutants

are temperature sensitive in that high temperature promotes dauer formation, but at low temperatures these animals can become reproductive adults (Riddle, Blumenthal et al. 1997).

Pathways regulating dauer entry including insulin, serotonin, TGF- β , and nuclear hormone receptor signaling also regulate fat storage (Figure 1). *C. elegans* defective in insulin signaling enter the dauer stage constitutively and have increased fat stores as dauers or adults (Kimura, Tissenbaum et al. 1997; Riddle, Blumenthal et al. 1997). *C. elegans* have approximately 40 insulin-like peptides, several of which are implicated in dauer formation (Pierce, Costa et al. 2001; Li, Kennedy et al. 2003). Insulin signaling is stimulated by DAF-28, which is expressed in the ASI and ASJ ciliated sensory neurons (Li, Kennedy et al. 2003). Expression of *daf-28* is downregulated in dauer animals produced by either starvation or pheromone treatment (Li, Kennedy et al. 2003). In contrast, *INS-1* antagonizes insulin signaling and overexpression of mammalian or *C. elegans ins-1* promotes dauer entry (Pierce, Costa et al. 2001). The insulin receptor, DAF-2, signals through the phosphoinositide 3-kinase, AGE-1, and protein kinases PDK-1, AKT-1, and AKT-2 (Morris, Tissenbaum et al. 1996; Paradis and Ruvkun 1998; Paradis, Ailion et al. 1999). Together AKT-1 and AKT-2 inhibit nuclear localization of DAF-16, a FOXO transcription factor, which suppresses dauer formation and fat storage phenotypes of *daf-2* mutants (Figure 1) (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997; Lin, Hsin et al. 2001). Selective expression of DAF-2 in neurons or muscle rescues the increased fat storage phenotype of *daf-2* mutants (Apfeld and Kenyon 1999; Wolkow, Kimura et al. 2000). Insulin signaling represents a conserved pathway in *C. elegans* and mammals that regulates energy homeostasis.

The TGF- β pathway also regulates entry into the dauer state. The *daf-7*/TGF- β ligand is expressed in the ASI ciliated sensory neurons (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). Ablation of ASI neurons leads to constitutive dauer formation, presumably due to lack of *daf-7*/TGF- β signaling (Bargmann and Horvitz 1991). Transcriptional levels of *daf-7::gfp* are decreased in the presence of high temperature or dauer pheromone (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). In contrast, food promotes expression of a *daf-7::gfp* reporter (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). Thus, *daf-7* regulates dauer formation by acting as a gauge of environmental conditions. DAF-7 binds to a heteromeric complex of type I TGF- β receptor, DAF-1, and a type II TGF- β receptor, DAF-4 (Georgi, Albert et al. 1990; Estevez, Attisano et al. 1993). In the active ligand-receptor complex, DAF-4 phosphorylates and activates DAF-1 (Savage-Dunn 2001). Activated DAF-1 receptor phosphorylates SMAD transcription factors encoded by *daf-14* and *daf-8* in *C. elegans* (Inoue and Thomas 2000), which can enter the nucleus and regulate transcription (Savage-Dunn 2001). Loss of function mutations in *daf-7*, *daf-1*, *daf-4*, *daf-14*, or *daf-8* result in constitutive dauer formation. In contrast, the *daf-3* Co-SMAD suppresses the dauer constitutive phenotype of the above mutants (Patterson, Kowalik et al. 1997). This TGF- β signaling cascade regulates entry into dauer state in response to external environmental conditions (Figure 1). Dauer animals lacking the TGF- β ligand, *daf-7*, have increased fat stores, and do not require *daf-16*/FOXO signaling to accumulate excess fat (Kimura, Tissenbaum et al. 1997; Ogg, Paradis et al. 1997).

In *C. elegans*, serotonin is thought to signal the presence of food (Horvitz, Chalfie et al. 1982; Sawin, Ranganathan et al. 2000). Animals defective in serotonin synthesis

have a mild dauer constitutive phenotype and increased fat stores (Sze, Victor et al. 2000). Both the dauer formation and fat storage are partially dependent on the TGF- β pathways since mutation in *daf-3* partially suppress these phenotypes (Sze, Victor et al. 2000). The role of serotonin signaling in obesity may be conserved as mice lacking serotonin receptor 5HT2C are hyperphagic and overweight (Tecott, Sun et al. 1995).

Downstream of insulin and TGF- β signaling, dauer formation is regulated by sterol hormone signaling pathways. As dauers, mutants in a cytochrome P450, *daf-9*, also have increased fat stores (Gerisch, Weitzel et al. 2001; Gerisch and Antebi 2004). DAF-9 processes a sterol ligand for the nuclear hormone receptor DAF-12 (Jia, Albert et al. 2002; Motola, Cummins et al. 2006). Loss of function mutations in *daf-12* suppress the dauer formation and fat storage phenotype of *daf-9* mutants (Gerisch, Weitzel et al. 2001). Surprisingly, although loss of function mutations in *daf-12* suppress the dauer formation phenotype of insulin and TGF- β pathway mutants, it does not suppress fat accumulation (Riddle, Blumenthal et al. 1997; Gerisch, Weitzel et al. 2001). Although dauer animals have increased fat, the insulin and TGF- β pathways also regulate fat stores independently of dauer formation.

Additional fat storage pathways in *C. elegans*

Recently, researchers have described several non-dauer genes that also affect fat accumulation in *C. elegans*. Key mammalian metabolic regulators such as the transcription factor SREBP, *shp-1*, also regulate fat metabolism in *C. elegans* (McKay, McKay et al. 2003; Yang, Vought et al. 2006). In both *C. elegans* and mammals SREBP regulates expression of important metabolic enzymes including fatty acid synthase, acyl-CoA carboxylase, and stearoyl-CoA desaturases (McKay, McKay et al. 2003; Yang,

Vought et al. 2006). Animals with defects in fatty acid synthesis have decreased fat storage along with slow growth and reduced fertility (Yang, Vought et al. 2006; Brock, Browse et al. 2007).

Mutations in *C. elegans* homologues of tubby and bardet-biedl syndrome genes result in increased fat storage as assayed by increased accumulation of the vital lipophilic dyes Nile Red and BODIPY (Mukhopadhyay, Deplancke et al. 2005; Mak, Nelson et al. 2006). Mice lacking *tubby* have adult onset obesity and insulin resistance in addition to cochlear hair cell degeneration (Carroll, Gomez et al. 2004). TUBBY is expressed throughout the brain, and researchers have suggested that it could interact with satiety pathways (Carroll, Gomez et al. 2004). In humans, mutations in BBS genes result in obesity in addition to defects in kidney development and right-left asymmetry. BBS proteins function in microtubule based processes (Bisgrove and Yost 2006). In *C. elegans*, BBS genes are expressed in ciliated sensory neurons and are necessary for fully functional cilia (Blacque, Reardon et al. 2004). Both *tubby* and BBS genes are thought to function in *C. elegans* ciliated neurons to regulate fat storage (Mukhopadhyay, Deplancke et al. 2005; Mak, Nelson et al. 2006). This increase in fat stores in *tub-1* and BBS mutants does not require FOXO/*daf-16* insulin signaling, but for *tub-1* is dependent on a Rab-GTPase *rgb-3* (Mukhopadhyay, Deplancke et al. 2005; Mak, Nelson et al. 2006).

An RNAi based genome-wide screen identified about 400 additional genes that alter accumulation of nile red (Ashrafi, Chang et al. 2003). Genes included in this set are involved in metabolism, signal transduction, transcription, and other cellular processes (Ashrafi, Chang et al. 2003). How these genes alter metabolism in *C. elegans* is currently

under investigation. *C. elegans* has many conserved pathways that regulate fat storage, yet understanding the behavioral or metabolic adaptations underlying regulation of fat storage will be key to establishing *C. elegans* as a model for studying obesity.

Feeding behavior in *C. elegans*

In the laboratory, *C. elegans* feed on bacteria using a neuromuscular pump called the pharynx. The pharynx brings in bacteria through the corpus and filters out the bacteria from surrounding liquid. The bacteria are then transported from the corpus to the isthmus, after which the grinder of the terminal bulb grinds the bacteria and propels it into the gut (Figure 2A-B) (Albertson and Thomson 1976). This pharyngeal pumping, or feeding behavior, is rhythmic and occurs over 200 times per minute. This is accomplished through the coordinated action of 20 muscles and 20 associated pharyngeal neurons (Albertson and Thomson 1976). The pharyngeal nervous system does not synapse onto the other 282 neurons found in an adult hermaphrodite (White, Southgate et al. 1986).

In order to understand the role of the pharyngeal nervous system in regulating pharyngeal pumping, researchers use a combination of neuron ablation, electrophysiology, and genetic techniques. Ablation of only one pharyngeal neuron, M4, abolishes pharyngeal pumping. In the absence of the M4 neuron, animals are unable to move bacteria from the corpus through the isthmus (Figure 2C). These animals fail to grow and reach maturity (Avery and Horvitz 1987). Additional neuron ablation studies highlighted that the MC neuron is required for rhythmic fast pumping (Avery and Horvitz 1989). Individual or combinatorial ablations of other pharyngeal neurons did not dramatically alter pharyngeal pumping behavior. Researchers have proposed that if other

neurons play a role in pharyngeal pumping it will be to modulate pharyngeal pumping in response to environmental conditions or other behavioral programs (Avery and Horvitz 1989). Electrical recording of pharyngeal activity, or electropharyngeograms (EPG's), have been used to further understand the role of several pharyngeal neurons. Based on these studies, the MC motor neuron was defined as an excitatory neuron, while the M3 motor neuron is required for rapid transition between the excitatory contracted phase and the inhibitory relaxed phase of pharyngeal pumping (Raizen and Avery 1994).

Genetic screens for modulators of pumping rate have complemented the neuronal ablation studies. Mutants in pharyngeal pumping were isolated based on a starved appearance presumably due to defects in food transport (Avery 1993). The defects of these “eat” mutants are likely to be mechanical defects in food transport, rather than sensory defects since the mutants isolated in this screen did not exhibit the dauer or egg-laying phenotypes of animals defective in sensing food (Avery 1993). These mutations highlight the importance of several neurotransmitters in pharyngeal pumping. Mutations in a subunit of a nicotinic acetylcholine receptor, *eat-2*, result in decreased pharyngeal pumping rate accompanied by impaired growth. These cholinergic receptors cluster in the pharyngeal muscle postsynaptically to projections from the excitatory cholinergic MC neuron (McKay, Raizen et al. 2004). Similarly, mutations in a vesicular glutamate transporter, *eat-4*, also disrupt pharyngeal pumping. *eat-4* is expressed in the M3 pharyngeal neuron and EPG analysis shows that mutations in *eat-4* phenocopy ablation of the M3 pharyngeal neuron. These data suggest that M3 regulates pharyngeal pumping by secreting glutamate (Raizen and Avery 1994; Lee, Sawin et al. 1999).

Several other neurotransmitters such as octopamine and serotonin also regulate pharyngeal pumping but in a modulatory manner. Exogenous addition of octopamine decreases pharyngeal pumping rate while addition of serotonin increases pharyngeal pumping rate (Horvitz, Chalfie et al. 1982). Little is known about how octopamine regulates pharyngeal pumping. Additionally, the pair of interneurons that synthesize octopamine are not connected to the pharyngeal nervous system suggesting that octopamine may regulate pharyngeal pumping as a neurohormone (Alkema, Hunter-Ensor et al. 2005). Serotonin is synthesized in a pair of pharyngeal neurons, NSM (Horvitz, Chalfie et al. 1982) which is predicted to secrete serotonin in response to food (Albertson and Thomson 1976; Avery and Thomas 1997). Serotonin increases the rate of pharyngeal pumping by enhancing the activity of the inhibitory glutamatergic M3 neuron at the same time that it enhances the activity of the excitatory cholinergic MC neuron (Figure 2C). By stimulating both M3 and MC, serotonin speeds up the contraction-relaxation cycle of pharyngeal pumping (Niacaris and Avery 2003).

Pharyngeal pumping is also modulated by environmental factors such as food availability, mechanical stimuli, and mating (Horvitz, Chalfie et al. 1982; Avery and Horvitz 1990; Liu and Sternberg 1995; Keane and Avery 2003). In the absence of food, *C. elegans* pump at a reduced rate. In addition, starved animals will initiate pumping behavior at lower concentrations of bacteria than well-fed animals (Avery and Horvitz 1990). Mutants in the *C. elegans* CAPS protein, *unc-31*, which is required for dense core vesicle fusion, fail to respond to starvation by downregulating pharyngeal pumping (Avery, Bargmann et al. 1993). *unc-31* is required for secretion of neuropeptides, insulin, and biogenic amines, suggesting that the nervous system is required for environmental

factors to modulate the rate of pharyngeal pumping. Animals treated with exogenous serotonin or animals lacking tyramine and octopamine also fail to downregulate pharyngeal pumping in the absence of food (Horvitz, Chalfie et al. 1982; Alkema, Hunter-Ensor et al. 2005).

Other environmental factors also influence pharyngeal pumping. Harsh mechanical stimuli in the form of a tail tap will inhibit pharyngeal pumping through glutamate gated chloride channels (Keane and Avery 2003). Adult animals may downregulate pharyngeal pumping in response to mechanical stimuli as part of an escape response from predators. Other behaviors also affect pharyngeal pumping rate. For example, mating males downregulate pharyngeal pumping in a process that is dependent on the serotonergic NSM neuron (Liu and Sternberg 1995; Gruninger, Gualberto et al. 2006). Pharyngeal pumping in *C. elegans* is a dynamic behavior regulated by food availability as well as other behavioral programs. While we understand a significant amount about the neurons and neurotransmitters required for pharyngeal pumping, the genes or neurons that underlie the response of pharyngeal pumping to changing environmental conditions remain unexplored. Furthermore, it is unclear if *C. elegans* coordinately regulates feeding behavior and fat storage.

Thesis Summary

Here we use the *C. elegans* TGF- β pathway as a paradigm for exploring the relationship between feeding behavior and fat storage. TGF- β signaling coordinates external conditions such as population density and food availability with development, metabolism, and behavior. Mutants in the *daf-7*/TGF- β pathway have increased fat stores, constitutive dauer formation, and reduced feeding rate. We find that the TGF- β ligand *daf-7* signals to the *daf-1* receptor in two interneurons, RIM and RIC, to regulate dauer formation, pharyngeal pumping, and fat storage. These two neurons are the site of tyramine and octopamine synthesis in *C. elegans*. Interestingly, we find that tyramine and octopamine function downstream of TGF- β signaling to regulate pharyngeal pumping, yet do not affect fat storage or dauer formation. Fat storage is promoted by G-protein signaling, specifically through metabotropic glutamate receptors. Mutations in two metabotropic glutamate receptors, *mgl-1* and *mgl-3*, suppress the fat storage phenotype of *daf-7* mutants; however, these mutations do not affect pharyngeal pumping rate or dauer formation. Thus, while TGF- β signaling in two neurons regulates feeding, fat storage, and dauer formation, distinct molecular pathways emanating from these neurons function to regulate each of these physiologies.

We also identify 20 additional genes that affect fat storage and pharyngeal pumping behavior. These genes are predominantly expressed in the pharyngeal muscle and neurons, as well as head neurons. Here we investigate the interaction between these newly identified feeding regulatory genes and the serotonergic, TGF- β , and glutamate signaling pathways, which regulate pharyngeal pumping. This work suggests a complex

relationship between feeding behavior and fat storage in *C. elegans*. We identify specific genetic and neural circuits operating in animals to coordinately regulate feeding behavior and fat storage in anticipation of changing environmental conditions.

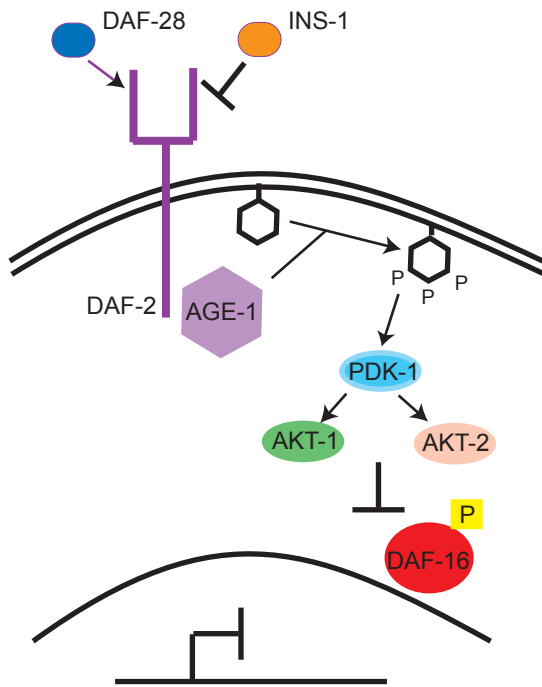
Figure 1: Pathways regulating dauer formation and metabolism in *C. elegans*

The insulin signaling pathway regulates dauer formation through the DAF-2 insulin receptor, which is activated by DAF-28 and inhibited by INS-1 in addition to other insulin peptides. Signaling through DAF-2 activates the PI3 kinase AGE-1 resulting in the accumulation of PIP3 in the membrane, which activates kinases PDK-1, AKT-1, and AKT-2. AKT-1 and AKT-2 act in a redundant manner to inhibit nuclear accumulation of FOXO transcription factor DAF-16; thus preventing transcription of DAF-16 target genes.

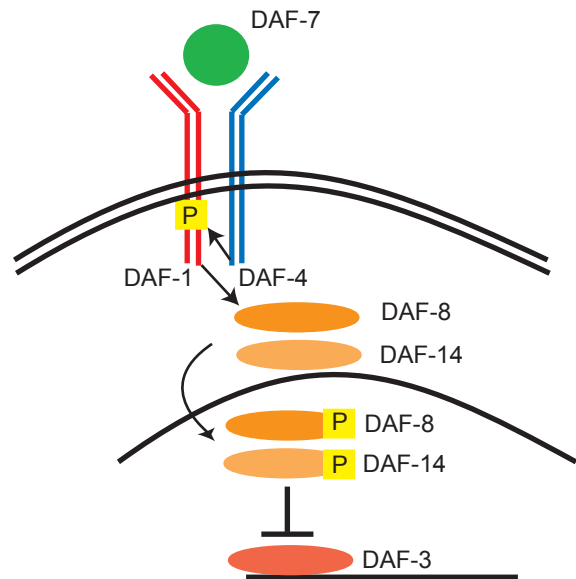
The DAF-7 TGF- β ligand binds to transmembrane serine/threonine kinases receptors, DAF-1 and DAF-4. Active TGF- β signaling results in phosphorylation of DAF-1 by DAF-4. DAF-1 in turn phosphorylates SMAD transcription factors DAF-8 and DAF-14 resulting in nuclear accumulation and inhibition of DAF-3 Co-SMAD.

The cytochrome P450 DAF-9 functions in XXX or hypodermal cells to modify a sterol hormone, which binds to and regulates the activity of the nuclear hormone receptor DAF-12 in a non-cell autonomous manner. Active DAF-12 leads to dauer formation through transcription of target genes.

A Insulin Signaling



B TGF-beta Signaling



C Lipophilic Hormone Signaling

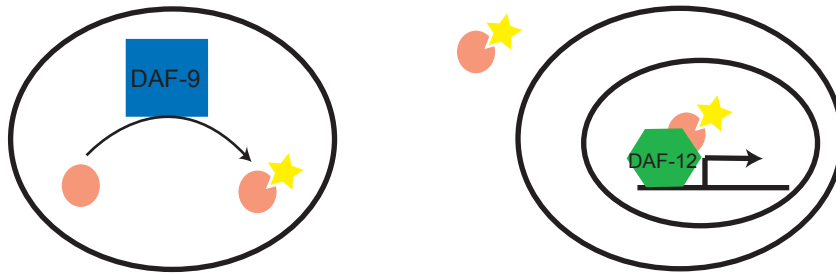
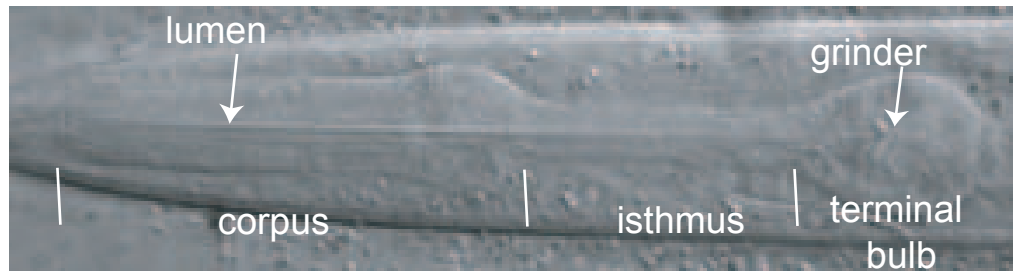


Figure 2: Regulation of pharyngeal pumping in *C. elegans*

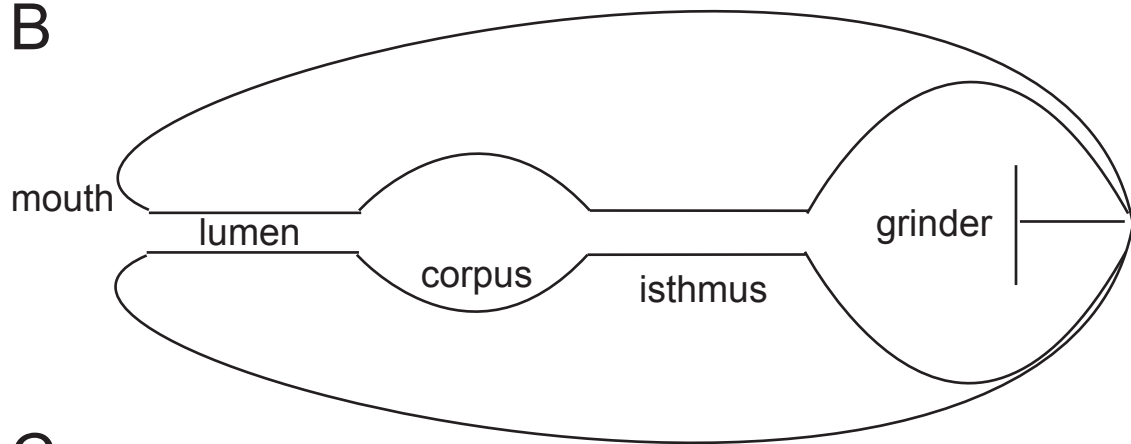
(A-B) DIC (A) and cartoon (B) image of *C. elegans* pharynx. Food enters the mouth of the animals and moves through the lumen of the pharynx. The corpus filters bacteria from surrounding liquid. Bacteria moves through the isthmus by peristaltic waves of muscle activity and enters the terminal bulb where is crushed by the grinder and propelled into the gut.

(C) Regulation of *C. elegans* pharyngeal pumping by key neurons and neurotransmitters. Serotonin released from NSM activates GPCR's on pharyngeal motor neurons MC and M3. Activation of MC leads to release of the excitatory neurotransmitter acetylcholine, which binds to *eat-2* nicotinic acetylcholine receptor on pharyngeal muscle. M3 activation leads to release of the inhibitory neurotransmitter glutamate. Serotonin functions to increase pharyngeal pumping by increasing activity of both MC and M3 thus speeding up the cycle of muscle contraction. The M4 motorneuron is required for viability of *C. elegans* and regulates isthmus peristalsis perhaps using acetylcholine.

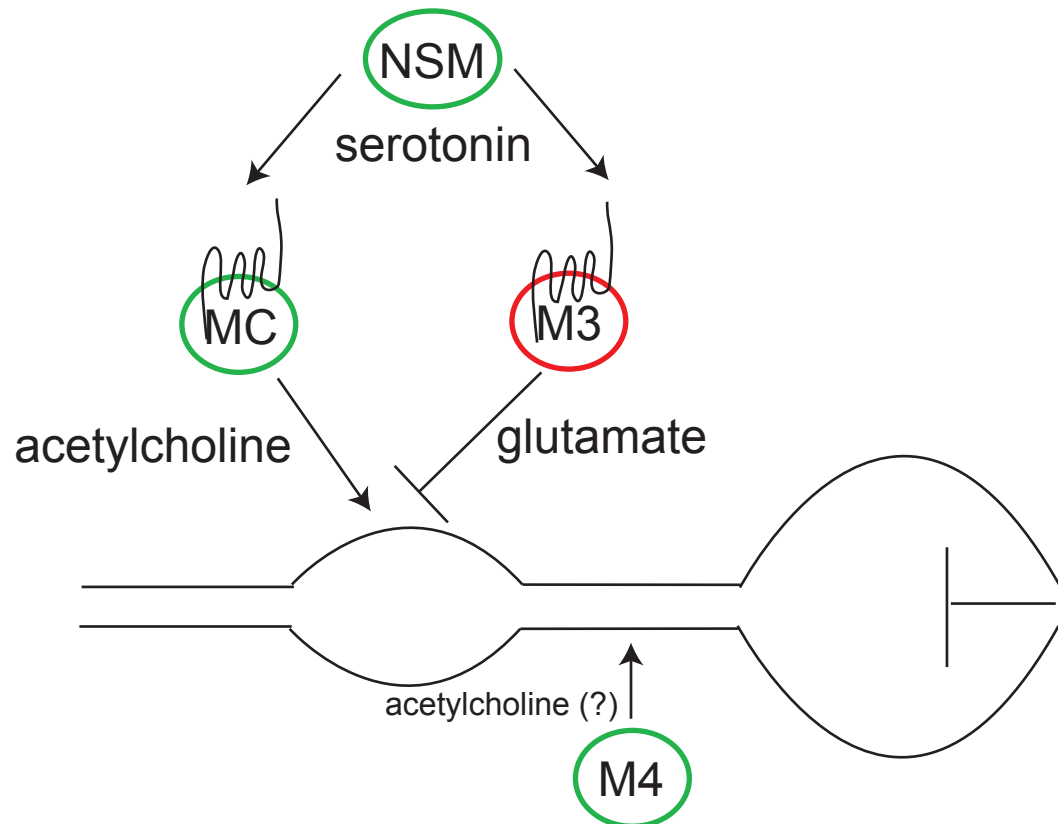
A



B



C



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**Chapter II: TGF- β signaling regulates feeding, fat storage, and dauer
formation in *C. elegans***

Introduction

All organisms tightly regulate energy homeostasis to maintain sufficient energy supplies despite changing environmental conditions. Mammals sense internal energy stores using peptides such as leptin, which are secreted from sites of fat storage and act in the brain to regulate both energy expenditure and feeding behavior (Flier 2004). In mammals, many molecules are known to act as internal sensors of energy stores; however, less is known about the role of changing environmental conditions in regulating metabolism and fat storage. Recent studies have implicated dopaminergic reward pathways in coordinating palatability of food with feeding behavior.

The complexity of mammalian neurocircuitry and food related cues have made it difficult to study molecular pathways that coordinate environmental conditions and internal physiology. The relative simplicity of *C. elegans* offers many advantages for understanding this question. *C. elegans* has a simple nervous system consisting of 302 neurons and is a well-suited model system for the study of complex behavioral circuits (de Bono and Maricq 2005). In addition, many *C. elegans* behaviors such as egg-laying, feeding, and foraging are modulated by food (Trent 1982; Avery and Horvitz 1990; Sawin, Ranganathan et al. 2000; Hills, Brockie et al. 2004). As in mammals, food is a strong motivator for *C. elegans* behavior. For example, *C. elegans* can learn to associate the presence or absence of food with environmental stimuli such as smell and temperature (Saeki, Yamamoto et al. 2001; Murakami, Bessinger et al. 2005). For these reasons, we use *C. elegans* as a model system to understand circuits coordinating environmental conditions with behavior and metabolism.

C. elegans monitor environmental conditions to decide whether to enter the long-lived dauer state or to continue to reproductive development. Dauer larvae are resistant to environmental stresses such as high temperature and can survive for extended periods of time without food. In addition, dauer larvae have increased fat stores, decreased feeding, and decreased energy expenditure (Wadsworth and Riddle 1989; Riddle, Blumenthal et al. 1997). Harsh environmental conditions such as high temperature, reduced food availability, and high population density promote entry into the dauer state (Riddle, Blumenthal et al. 1997). Molecular pathways that regulate entry into the dauer state coordinate internal physiology with external conditions (Riddle, Blumenthal et al. 1997). Insulin and TGF- β pathways regulate entry into the dauer state, metabolism, and lifespan (Kimura, Tissenbaum et al. 1997; Shaw, Luo et al. 2007). The TGF- β ligand, DAF-7, is an environmentally responsive neuroendocrine molecule that regulates metabolism and dauer formation (Ren, Lim et al. 1996; Kimura, Tissenbaum et al. 1997).

daf-7 is expressed in the ASI pair of sensory neurons and levels of *daf-7* expression change with environmental conditions (Ren, Lim et al. 1996). In times of low food or high population density, *daf-7* is transcriptionally repressed, leading animals to enter the dauer state (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). Mutants lacking *daf-7* also have adult phenotypes including egg-retention and increased aggregation on bacterial lawns, both of which are phenotypes reminiscent of animals entering a starved state (Trent 1982; de Bono, Tobin et al. 2002). Feeding behavior, or pharyngeal pumping, is also regulated by food availability. *C. elegans* feed using a neuromuscular pump called the pharynx: the rate of pharyngeal pumping correlates with amount food intake and is entrained by the experience of starvation (Avery and Horvitz

1990). With respect to dauer formation, egg-laying, and aggregation, animals lacking TGF- β signaling display phenotypes of animals preparing to enter a starved state. We find that mutants in the *daf-7*/TGF- β pathway also have defects in pharyngeal pumping and have increased fat stores. We show that the pharyngeal pumping, fat storage, and egg-laying phenotypes are not developmental, but can be affected by the environment in adults as well as larval animals. Together, these data suggest that DAF-7 is a component of a regulatory network that coordinates food availability or population density with metabolism and feeding and egg-laying behaviors of the animal.

Results

Decreased TGF- β signaling causes increased fat storage and decreased feeding rate

The DAF-7 TGF- β ligand is secreted from ASI sensory neurons and binds to heterodimeric type I and type II TGF- β receptors, DAF-1 and DAF-4 (Figure 1A). Activation of the two receptors leads to phosphorylation of transcription factors called SMADS. Activation of DAF-1 and DAF-4 leads to inhibition of the DAF-3 co-SMAD; thus, mutations in DAF-3 suppress the dauer formation and egg-laying phenotypes of TGF- β ligand and receptor mutants (Figure 1A) (Trent, Tsuing et al. 1983; Patterson, Kowook et al. 1997). The nuclear hormone receptor DAF-12 functions downstream of several dauer pathways, including TGF- β and insulin signaling, regulating entry into dauer (Figure 1A) (Antebi, Yeh et al. 2000).

We examined the fat storage and pharyngeal pumping phenotypes of *daf-1* and *daf-7* mutant non-dauer animals by growing animals at 20°C. We find that mutations in *daf-7* or *daf-1* decrease pharyngeal pumping by 20-25% and increase fat storage ~2.5 fold as monitored by Sudan Black B staining. While mutations in the co-SMAD *daf-3*

suppress both the fat storage and pharyngeal pumping phenotypes of *daf-1* mutants, loss of the nuclear hormone receptor *daf-12* does not (Figure 1B-D). Thus, dauer formation can occur independently of changes in fat storage or pharyngeal pumping.

C. elegans respond to starvation by reducing the rate of pharyngeal pumping by ~50% (Figure 1B). Both *daf-7* and *daf-1* mutants respond to starvation by reducing pharyngeal pumping rates to the same level as wild type. *daf-3* and *daf-12* mutant animals respond to starvation like wild type (Figure 1B).

TGF- β signaling regulates pharyngeal pumping and fat storage in a non-developmental manner

In *C. elegans* TGF- β signaling has been studied as a developmental pathway regulating dauer decision. However, TGF- β mutants have many adult phenotypes including egg-laying, bordering, fat storage, and pharyngeal pumping suggesting the pathway may also function in a non-developmental manner. Dauer pheromone is constitutively secreted as a gauge of population density and functions in part by downregulating *daf-7* in ASI. We applied dauer pheromone to adult animals to determine whether the TGF- β pathway functions to regulate pharyngeal pumping in response to changing environmental conditions. Addition of dauer pheromone to adults resulted in a small but significant decrease in pharyngeal pumping rate (Figure 1B). This decrease is likely to be due to changes in the TGF- β pathway since mutation in *daf-7*, *daf-1*, or *daf-3* suppress the effects of pheromone on pharyngeal pumping (Figure 1B). We did not observe any changes in fat storage when applying dauer pheromone to adults (data not shown).

In order to determine if fat storage could also be modulated by the TGF- β pathway in a non-developmental manner, we rescued *daf-1(m40)* animals by expressing the *daf-1* open reading frame (ORF) under the control of a heat shock inducible promoter, *hsp-16.2* (Stringham, Dixon et al. 1992; Bacaj and Shaham 2007). Without heat shock to induce *daf-1* expression, *daf-1[phsp-16.2::daf-1]* transgenic animals phenocopy *daf-1* mutant animals with respect to dauer formation, pharyngeal pumping, and fat storage (Figure 2B-C). A brief heat shock was used to induce *daf-1* expression and 24 hours after induction *daf-1[phsp-16.2::daf-1]* animals have reduced fat stores and wild type pharyngeal pumping rates (Figure 2A-C). In contrast, non-transgenic *daf-1* animals are unaffected by heat shock treatment (Figure 2B-C). We observed changes in pharyngeal pumping rates 30 minutes after *daf-1* induction, suggesting that pharyngeal pumping may be modulated on a faster time scale than metabolism. Furthermore we found that expression of *daf-1* in adult animals also abrogates the egg-retention phenotype of *daf-1(m40)* animals (Figure 2D). Addition of pheromone to decrease TGF- β signaling in adults and heat shock induction of *daf-1* to rescue TGF- β signaling in post-dauer animals both demonstrate that the *daf-7*/TGF- β functions as a gauge of environmental conditions throughout the life of *C. elegans*, not just during the L1 dauer decision.

Non-dauer TGF- β signaling pathways do not regulate fat storage or pharyngeal pumping

In addition to the *daf-7*/TGF- β pathways, the TGF- β ligand *dbl-1* regulates body size, while the TGF- β ligand *unc-129* is involved in axon guidance (Suzuki, Yandell et al. 1999; Patterson and Padgett 2000). We find that mutations in *dbl-1* or *unc-129* do not affect pharyngeal pumping rates (Figure 3A). The TGF- β ligand *dbl-1* binds to the type I

receptor *sma-6* and type II receptor *daf-4*, which is shared with the *daf-7*/TGF- β pathway (Krishna, Maduzia et al. 1999). Mutations in *daf-4* result in a decrease in pharyngeal pumping, consistent with its role in the *daf-7*/TGF- β pathway (Figure 3A). The receptor *sma-6*, which functions only in regulating body size does not have a pharyngeal pumping phenotype (Figure 3A). In addition, mutations in SMAD protein *sma-4* do not affect pharyngeal pumping rates.

In *C. elegans* the vital dye nile red can be used to visualize some fat droplets in the intestine (Ashrafi, Chang et al. 2003). Mutation in *daf-7* results in decreased and non-punctate nile red staining (Figure 3B). However, mutations in *dbl-1*, *sma-6*, or *sma-2* do not alter the nile red staining pattern in the same manner as *daf-7(e1372)* animals (Figure 3C). Together these results suggest that TGF- β pathways regulating body size or axon guidance do not function in the *daf-7*/TGF- β pathway to regulate fat storage or feeding behavior. Interestingly, although there may be some evidence to suggest that increased nile red staining correlates with increased fat stores (Ashrafi, Chang et al. 2003). Here we observe that animals mutant in *daf-7* have a decreased intensity of nile red staining, yet are high fat by sudan black staining and biochemical methods. The relationship between nile red intensity and fat storage is an active area of investigation in the laboratory.

Discussion

The canonical TGF- β signaling pathway regulates pharyngeal pumping and fat storage

Here we show that the *daf-7*/TGF- β pathway acts in non-developmental manner to regulate pharyngeal pumping and fat storage. Mutations in TGF- β receptors *daf-1* and

daf-4 or ligand *daf-7* result in increased fat storage and decreased pharyngeal pumping. This pathway is distinct from other TGF- β pathways regulating body size or axon guidance. As with the other phenotypes of *daf-1* and *daf-7* mutants, the co-SMAD *daf-3* functions downstream to regulate pharyngeal pumping and fat storage (Figure 4B). Fat storage and pharyngeal pumping are molecularly distinct pathways from dauer formation. The nuclear hormone receptor *daf-12* governs the entry into dauer state downstream of *daf-1* and *daf-7*; however, this pathway does not regulate fat or pharyngeal pumping phenotypes (Figure 4A). This is consistent with the TGF- β pathway regulating these phenotypes in a non-dauer dependent manner (Figure 4A). In the past, the *C. elegans* TGF- β pathway was studied as a developmental pathway regulating the entry into the dauer state. However, we show that signals affecting *daf-7* such as pheromone also function in adults to regulate pharyngeal pumping through the TGF- β pathway. In addition, *daf-1* expression after the dauer decision point will rescue the fat storage, feeding, and egg-laying defects of *daf-1(m40)* animals (Figure 4B). Together these data suggest that the TGF- β pathways functions in young animals and adults as a gauge of environmental conditions.

TGF- β signals the presence of food

Recent studies have implicated the TGF- β pathway in a wide range of adult phenotypes such as ageing, hyperoxia avoidance, bordering, and associative learning (de Bono, Tobin et al. 2002; Murakami, Bessinger et al. 2005; Chang, Chronis et al. 2006; Shaw, Luo et al. 2007). Many of these studies suggest that *daf-7*/TGF- β signals the presence of food or non-stressful conditions. Animals lacking food retain eggs, enter dauer, conserve energy, and feed less (Trent 1982; Golden and Riddle 1984; Avery and

Horvitz 1990; Van Gilst, Hadjivassiliou et al. 2005). These phenotypes are also present in *daf-1* or *daf-7* mutant animals.

In addition, wild type animals avoid hyperoxia (high oxygen concentrations) only when cultivated without food (Chang, Chronis et al. 2006). In contrast, animals mutant in *daf-7* will avoid hyperoxia in the presence or absence of food, again indicating that *daf-7* mutants behave starved or display an inability to correctly monitor external conditions (Chang, Chronis et al. 2006). *daf-7* mutants also fail to associate temperature with starvation in an associative learning paradigm, again suggesting a defect in food perception (Murakami, Bessinger et al. 2005). Similarly, we observed that wild type animals removed from food decrease their pumping rate to 47% of well-fed animals, while *daf-7* animals only decrease their pumping rate by 24%. Serotonin is also thought to signal the presence of food and a similar phenotype has been observed for animals defective in serotonin synthesis (Sze, Victor et al. 2000).

Conservation of TGF- β signaling

Here we show that the *daf-7* TGF- β ligand acts to coordinate environmental conditions with fat storage, dauer formation, and feeding behavior. The role of TGF- β signaling in energy homeostasis in mammals is not well defined. However, TGF- β is secreted from adipocytes in proportion to leptin and the amount of fat stores (Porreca, Di Febbo et al. 2002; Fain, Tichansky et al. 2005). Furthermore, overexpression of human TGF- β 1 in mice results in decreased fat storage and adipogenesis (Clouthier, Comerford et al. 1997). These data suggest that TGF- β signaling could be a conserved pathway for the regulation of energy homeostasis. In mammals, TGF- β signaling acts as an internal monitor of nutrients rather than a monitor of nutrients in the environment. The role of

mammalian TGF- β signaling in feeding behavior and fat storage will be an interesting area for future investigation.

Figure 1: TGF- β signaling modulates feeding rate and fat storage in adults.

(A) TGF- β signaling pathway regulating dauer formation in *C. elegans*.

(B) Pharyngeal pumping rates of TGF- β pathway components under various conditions. Well-fed, pheromone untreated: *daf-7(e1372)* and *daf-1(m40)* pumped at 81% and 77% of wild type rate, respectively. The reduced pumping rate of *daf-1(m40)* was suppressed by *daf-3(mgDf90)* but not by *daf-12(m20)*. Food deprived, pheromone untreated: upon food deprivation, pumping rates of young adult wild type and all mutant genotypes were reduced to the same rate (~50% well fed, wild type). Well fed, pheromone treated: pheromone treatment caused feeding reduction in well fed wild type and *daf-12(-)* animals. Similar treatment did not alter feeding rates of *daf-7*, *daf-1*, or *daf-3* mutants. Standard deviation bars are shown.

(C) Examples of Sudan Black B fat staining. Adult *daf-1(m40)* mutants have increased fat relative to wild type. This excess fat was suppressed by *daf-3(mgDf90)* but not *daf-12(m20)*.

(D) Quantitation of relative amounts of Sudan Black B staining. To compare fat levels between *daf-1(m40)* mutants and other genotypes, animals grown under identical conditions were fixed and stained using methods designed to minimize variation. For each genotype, average staining intensity of 10 animals is reported. Asterisks indicate statistical significance ($p < 0.001$) as determined by t-test when comparing genotype of interest with *daf-1(m40)*. Standard error bars are shown.

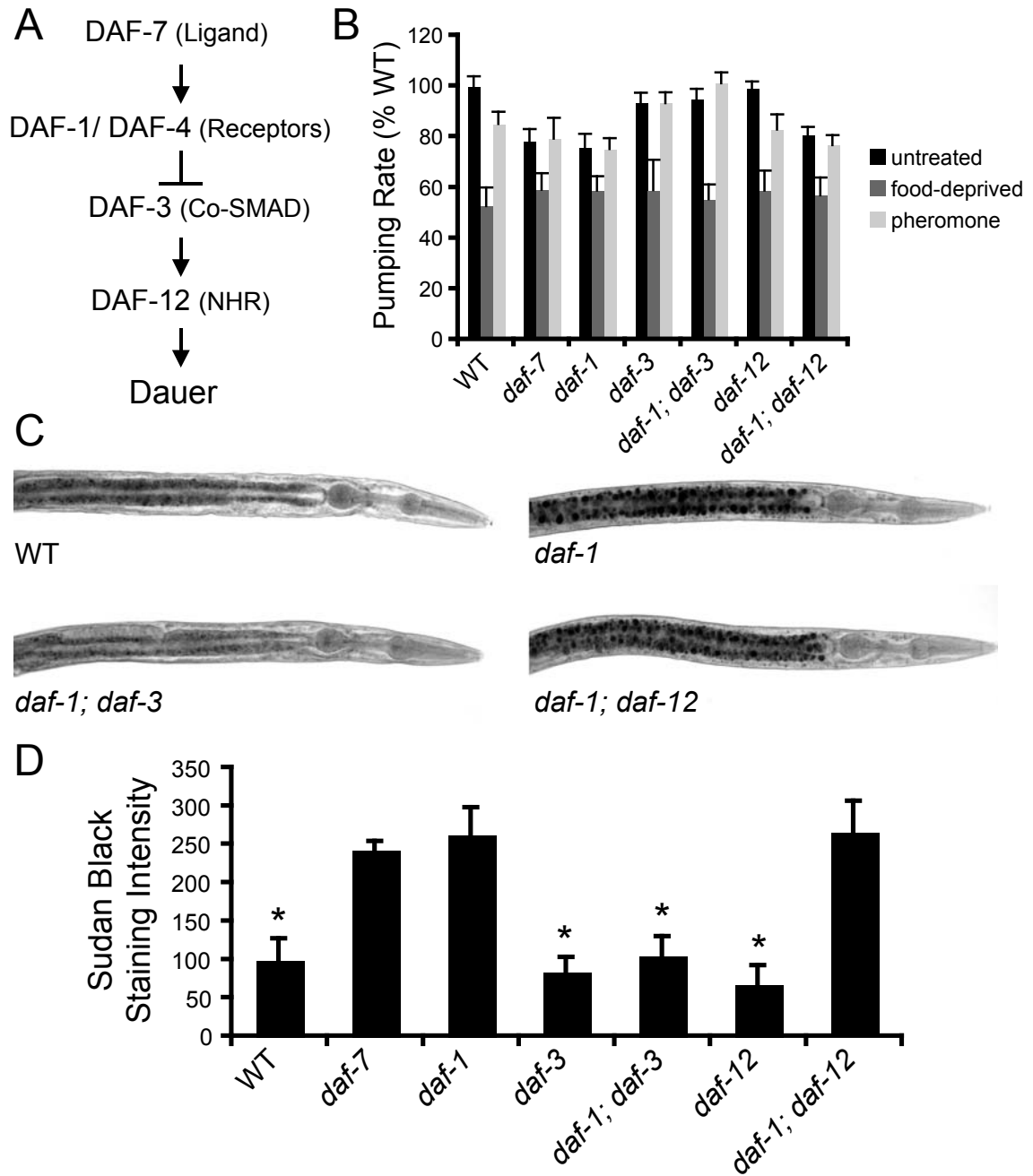


Figure 2: Activation of *daf-1* expression in late larval and adult stage animals rescues excess fat and reduced feeding rates of *daf-1(m40)* strain

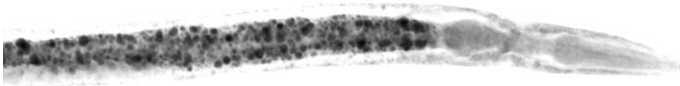
(A-B) Temporal activation of TGF- β signaling by placing *daf-1(+)* under the control of *phsp-16.2* heat shock promoter in *daf-1(m40)* animals resulted in reduced fat stores when animals were assayed 24 hours after a L3 heat shock. Expression of the *phsp-16.2::daf-1* construct did not affect the fat content of *daf-1(m40)* when *daf-1* expression was uninduced by heat shock. Asterisks indicate statistically significant changes ($p < 0.001$) as determined by t-test comparing transgenic animals and co-stained *daf-1(m40)* non-transgenic animals. Standard error bars are shown

(C) The reduction of pharyngeal pumping rate in *daf-1(m40)* animals was rescued by expressing *daf-1* under the heat shock promoter and inducing expression in adults.

Changes in pharyngeal pumping rate of *daf-1(-)* animals were noted 0.5h and 24h after induction of *daf-1*. Asterisks indicate statistically significant ($p < 0.001$) from *daf-1(m40)* as determined by ANOVA with Bonferroni post-test. Standard deviation bars are shown.

(D) Induction of *daf-1(+)* under the *hsp-16.2* promoter by heat shock reduces egg-retention phenotype of *daf-1(m40)* animals or *daf-1(m40); [phsp-16.2::daf-1]* transgenic animals where expression has not been induced by heat shock.

A

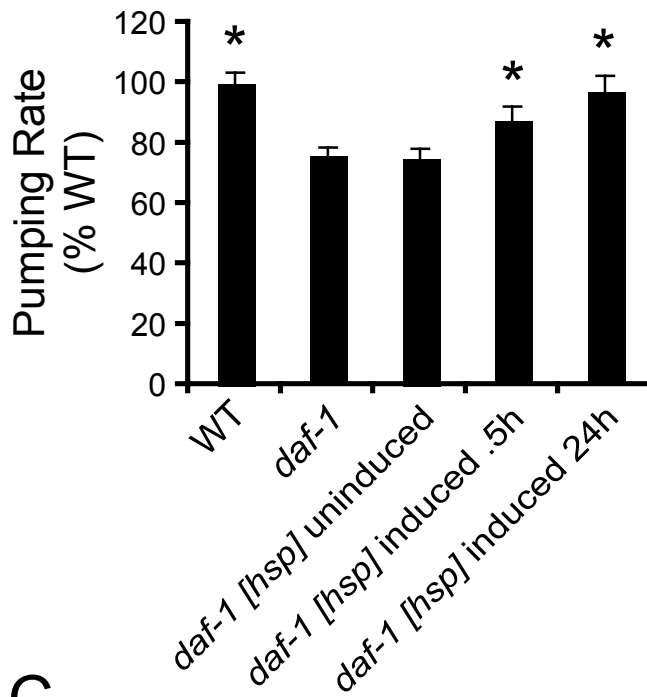


daf-1 heat shock control



daf-1 [*phsp-16.2::daf-1*]

B



C

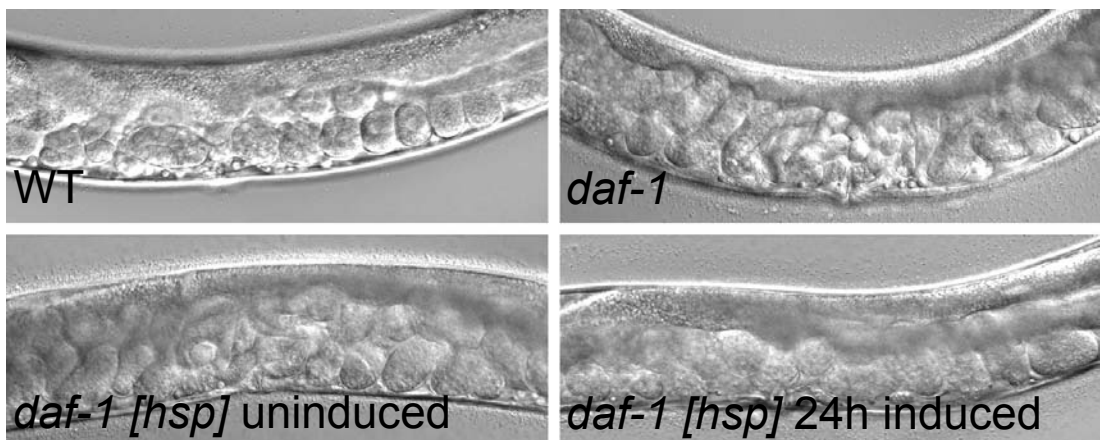
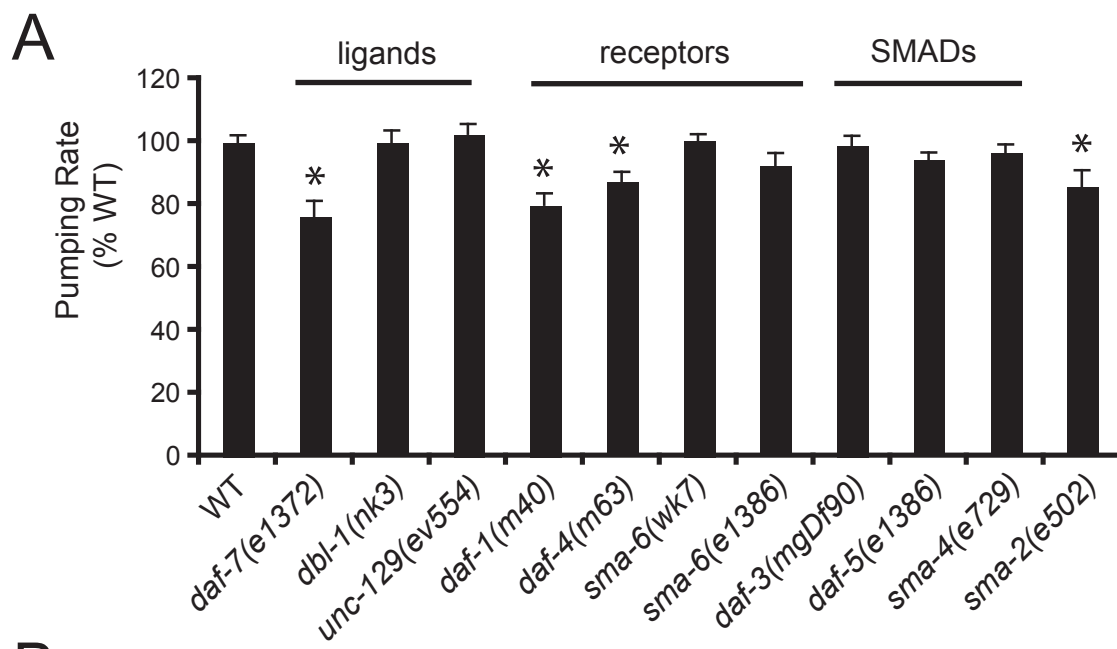


Figure 3: *dbl-1* and *unc-129* TGF- β pathways do not affect pharyngeal pumping or fat storage.

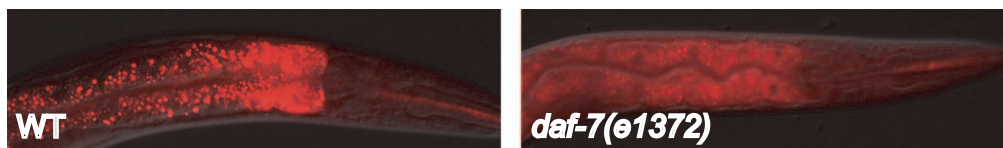
(A) Pharyngeal pumping phenotypes of *C. elegans* TGF- β pathways. Signaling components of the TGF- β pathways regulated dauer formation including the ligand *daf-7* and receptors *daf-1* and *daf-4* have decreased pharyngeal pumping rates. The TGF- β pathway that regulates body size in *C. elegans* including the ligand *dbl-1*, receptor *sma-6*, and smad *sma-4* have pharyngeal pumping rates that are similar to wild type. Mutations in the TGF- β ligand *unc-129* do not result in decreased pharyngeal pumping rates.

Asterisks indicate statistically significant ($p < 0.001$) from WT as determined by ANOVA with Bonferroni post-test. Standard deviation bars are shown.

(B-C) *daf-7(e1372)* animals have reduced and non-punctate Nile red staining compared to wild type animals. In contrast, mutations in *dbl-1*, *sma-6*, and *sma-2*, which regulate body size, have bright punctate Nile red staining similar to wild type.



B



C

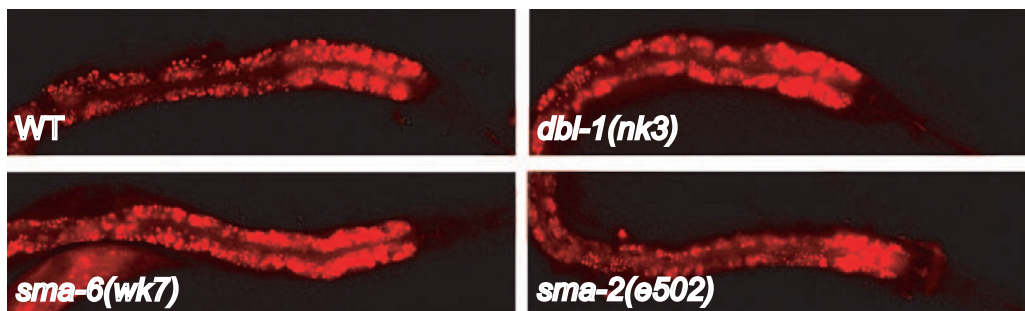


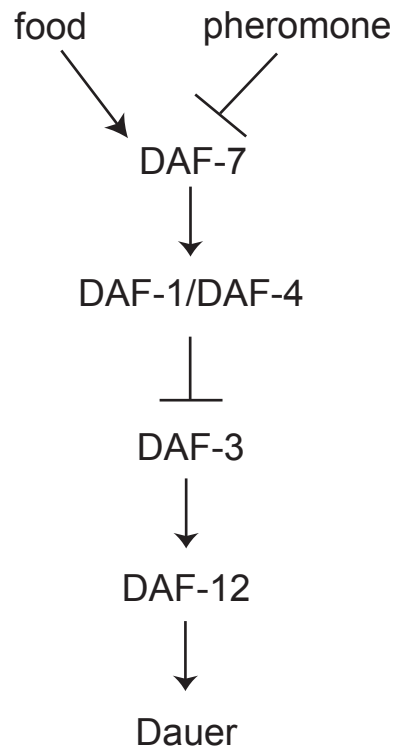
Figure 4: Model for TGF- β regulation of dauer formation, pharyngeal pumping, and fat storage.

(A) During the dauer decision in L1 larval stage animals low food and high pheromone inactivate DAF-7 resulting in inactivation of DAF-1 and DAF-4 TGF- β receptors. The active co-SMAD DAF-3 signals through the nuclear hormone receptor DAF-12 promoting entry into dauer state.

(B) After the dauer decision TGF- β signaling regulates pharyngeal pumping, fat storage, and egg-laying in a pathways that requires the co-SMAD DAF-3 but is independent of DAF-12.

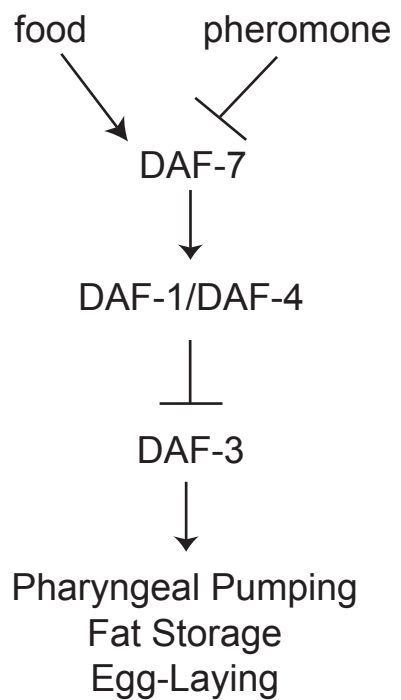
A

L1 Dauer Decision



B

Late Larval and Adult Animals



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**Chapter III: *daf-1* signaling in select interneurons regulates dauer formation,
feeding, and fat storage**

Introduction

Understanding obesity requires knowledge of key molecules and neuronal circuits. In mammals, researchers have identified important peptides and neurotransmitters that play a central role in regulating obesity. The sites of expression of neurotransmitters and peptide receptors have led to proposals regarding the specific neuronal circuits that underlie obesity (Balthasar 2006). The central circuit regulating energy homeostasis were thought to be the POMC and NPY/AgRP neurons of the arcuate nucleus because these neurons express the leptin receptor and other molecules key in regulating energy homeostasis. Leptin is a peptide hormone produced in adipose tissue in proportion to adiposity (Flier 2004). Loss of leptin or the leptin receptor leads to obesity (Zhang, Proenca et al. 1994; Tartaglia, Dembski et al. 1995). Leptin is thought to function by modulating the activity of POMC and NPY/AgRP neurons, suggesting that this could be a functional circuit to regulate feeding behavior and energy expenditure (Flier 2004; Cone 2005).

Recent studies highlight the importance of functionally testing the role of neuronal circuits proposed to underlie obesity (Balthasar 2006). Removing leptin receptors specifically from POMC neurons resulted in mild obesity, a striking contrast to the gross obesity of leptin receptor deletion mice. Researchers began to examine the role of other brain areas in regulating energy homeostasis (Balthasar, Coppari et al. 2004; Balthasar 2006). Additional studies identified other sites in the brain where the leptin receptor contributes to the regulation of energy homeostasis including the ventromedial nucleus and dopamine neurons of the midbrain (Dhillon, Zigman et al. 2006; Hommel,

Trinko et al. 2006). These studies suggest that, rather than a circuit involving only the arcuate nucleus, a distributed network of leptin receptors regulates energy homeostasis rather.

C. elegans is an ideal model system for identifying functional neuronal circuits given the relative ease of creating transgenic animals (Rankin 2002). The neuroendocrine TGF- β ligand *daf-7* regulates both feeding (or pharyngeal pumping) behavior and fat storage. In *C. elegans*, *daf-7* expression is restricted to the ASI pair of sensory neurons (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). *daf-7* is secreted from ASI and binds to two TGF- β receptors, *daf-1* and *daf-4*, which are broadly expressed in the nervous system (Gunther, Georgi et al. 2000). *daf-4* is expressed in additional tissues, including pharynx, intestine, and muscle; however, expression in these sites may be due to its function in an unrelated pathway governing body size. Using a series of cell specific rescue experiments, we have uncovered *C. elegans* neurons that require *daf-1* signaling to regulate fat storage and feeding. Circuit analysis reveals that *daf-1* is required in two interneurons, RIM and RIC, to regulate feeding behavior and fat storage.

Results

***daf-1* signaling in interneurons regulates pharyngeal pumping, fat storage, and dauer formation**

In order to identify the neuronal network underlying TGF- β signaling, we rescued *daf-1(m40)* by expression of the *daf-1* open reading frame, *daf-1(+)*, under ~20 neuron specific promoters. This experiment allowed us to define the cell(s) that require DAF-1 signaling for the proper regulation of pharyngeal pumping, fat storage, and dauer

formation. For each construct created to rescue *daf-1* mutants (*promoter::daf-1::gfp*), the same promoter was used to drive GFP (*promoter::gfp*) to validate the expression pattern (see examples in Figure 1A). We made multiple independent transgenic *C. elegans* lines for each rescue experiment (~3 lines/ construct) and results are consistent across these lines.

Expression of *daf-1(+)* under its own promoter or a pan-neuronal promoter, *egl-3*, rescues the dauer, pharyngeal pumping, and fat storage phenotypes of animals mutant in *daf-1* (Table 1, Figure 1A-C, Figure 3). These results are consistent with the previous experiments with the type II TGF- β receptor, *daf-4*, which established that neuronal expression of *daf-4* rescues the dauer formation phenotype of *daf-4* mutants (Inoue and Thomas 2000). In addition, expression of *daf-1(+)* under its own promoter, or using the *egl-3* promoter, rescued the egg-laying phenotype of *daf-1* mutants (Table 2 and Figure 2B-C).

We used additional promoters to rescue *daf-1(m40)* in ciliated sensory neurons, pharyngeal neurons, and interneurons to determine the sub-population of neurons which require DAF-1 signaling. We examined the role of ciliated sensory neurons because they have been implicated in the regulation of fat storage (Mukhopadhyay, Deplancke et al. 2005; Mak, Nelson et al. 2006). Expression of *daf-1(+)* in ciliated sensory neurons using the *bbs-1* and *osm-6* promoters did not rescue any *daf-1(m40)* phenotypes (Table 1, Figure 1 A-C, Figure 2B, Figure 3). In addition, *daf-1* expression in ASI alone did not rescue *daf-1* mutants, indicating that *daf-7* does not act in a cell autonomous manner. While ciliated neurons do play a role in the regulation of fat storage, *daf-1* probably functions downstream of sensory neurons.

DAF-1 could regulate pharyngeal pumping directly by modulating signaling in pharyngeal neurons; however, expression of *daf-1(+)* in these cells did not rescue the pharyngeal pumping or fat storage phenotypes (Table 1 and Figure 1B). Thus, *daf-1* does not affect pharyngeal pumping directly through the pharyngeal neurons. We also noticed *daf-1* expression in anterior pharyngeal glands. Using the B0280.7 promoter we expressed *daf-1(+)* in these cells, but we did not observe rescue of the pharyngeal pumping phenotype (Table 1). Expression of *daf-1(+)* in either pharyngeal neurons or sensory neurons is unable to rescue *daf-1(m40)* phenotypes. These data suggest that *daf-1* is likely to act in interneurons.

Expression of *daf-1(+)* under several interneuron promoters including *glr-1*, *glr-5*, and *glr-4* rescued the pharyngeal pumping, dauer formation, and fat storage phenotypes of *daf-1(m40)* animals (Table 1, Figure 1A-C, and Figure 3). These animals also had improved egg-laying behavior compared to *daf-1* mutants (Table 2). Surprisingly, expression under the *glr-2* interneuron promoter was not able to rescue *daf-1* mutants (Table 1). In addition, expression of *daf-1(+)* in GABAergic and cholinergic motor neurons using the *unc-47* and *unc-17* promoters did not rescue the pharyngeal pumping and fat storage phenotypes of *daf-1* mutants. Together, these data indicate that *daf-1* is required in a specific subset of interneurons to regulate pharyngeal pumping, fat storage, and dauer formation.

The *glr-1*, *glr-5* and *glr-4* promoters drive *daf-1(+)* expression in a common set of interneurons, AVA, RMD, and RIM (Table 3) (Brockie, Madsen et al. 2001). However, *daf-1(+)* expression under the *glr-2* promoter failed to rescue *daf-1* mutants. This promoter drives *daf-1(+)* expression in AVA and RMD but not RIM. This set of

overlapping interneuron promoters points to RIM as an important neuron for DAF-1 signaling (Table 3). We tested the specificity of this rescue by using more specific interneuron promoters to drive *daf-1(+)* expression.

***daf-1* is required in two interneurons, RIM and RIC to regulate dauer formation, pharyngeal pumping, and fat storage**

Using interneuron specific promoters we determined which interneurons require *daf-1(+)* expression to restore wild type pumping rates, fat storage, and dauer formation to *daf-1(m40)* animals. As suggested in the previous experiments, the *nmr-2* promoter which drives *daf-1(+)* expression in RIM and in 5 additional interneurons, rescues the pharyngeal pumping and fat phenotypes of *daf-1* mutants (Table 1). Furthermore, we used the *ggr-1*, *flp-1*, and *dop-1* promoters to express *daf-1(+)* in sets of interneurons that do not include RIM, these manipulations failed to rescue the *daf-1* mutants (Table 1).

We were able to take advantage of the *tdc-1* and *tbh-1* promoters to more directly test the role of RIM in *daf-1* signaling. Using the *tdc-1* promoter we expressed *daf-1(+)* in two interneurons RIM and RIC, and in the UV1 cells near the vulva of the animal (Alkema, Hunter-Ensor et al. 2005). Antibody staining showed that *tdc-1* is also expressed in the gonad sheath; however, we never observed this expression in transgenic animals (data not shown). Using the *tdc-1* promoter to drive *daf-1(+)* expression fully rescues pharyngeal pumping, fat storage, and dauer formation of *daf-1(m40)* animals. Surprisingly, expression of *daf-1(+)* under the *tbh-1* promoter, which expresses only in RIC, partially rescues these phenotypes (Table 1, Figure 1A-C, Figure 3).

The *pdaf-1::daf-1::gfp* construct co-localizes with *ptdc-1::RFP*, demonstrating that *daf-1* is expressed in both RIM and RIC (Figure 1D). Cell –specific rescue

experiments highlight the importance of *daf-1* signaling in either RIM or RIC for the regulation of pharyngeal pumping, fat storage, and dauer formation. However, additional cells may be involved in dauer formation, since expressing of *daf-1* in additional interneurons using the *unc-17* or *glr-8* promoters partially rescues the dauer constitutive phenotype (Table 1).

Co-SMAD *daf-3* also functions in RIM and RIC interneurons

Signaling through the TGF- β receptor *daf-1* leads to inhibition of the co-SMAD *daf-3*; thus mutations in *daf-3* suppress dauer, egg-laying, pharyngeal pumping, and fat storage phenotypes of *daf-1* or *daf-7* mutants. Because mutants in *daf-3* do not have any phenotypes on their own, we assayed the role of *daf-3* in RIM and RIC by expressing wild type genomic *daf-3*, *daf-3(+)*, in the *daf-1(m40); daf-3(mgDf90)* background. *daf-1(m40); daf-3(mgDf90)* animals are phenotypically wild type, so neurons expressing *daf-3(+)* will be mutant neurons in a wild type background. Expression of *daf-3(+)* under the *daf-1* pan-neuronal promoter reduced the pharyngeal pumping rate and increased fat stores of *daf-1(m40); daf-3(mgDf90)* animals (Table 4 and Figure 4A-C). As observed with the neuron specific *daf-1* rescue, expression of *daf-3(+)* in sensory neurons using the *osm-6* promoter, or in pharyngeal neurons using the *glr-7* promoter had no effect (Table 4 and Figure 4A-C). Interestingly, expression of *daf-3(+)* in RIM and RIC using the *tdc-1* promoter reduced pharyngeal pumping and increased fat storage of *daf-1(m40); daf-3(mgDf90)* animals (Table 4 and Figure 4A-C). However, rescue of *daf-3(+)* in RIC alone had no effect (Table 4 and Figure 4A-C). Expression of *daf-3(+)* under the *daf-1* or *tdc-1* promoters resulted in a moderate increase (20-40%) in the dauer constitutive

phenotype (Table 4). This suggests that TGF- β signaling in RIM and RIC is necessary for regulation of fat storage and pharyngeal pumping.

Although *daf-3* is a predicted SMAD transcription factor, cytoplasmic not nuclear localization of full-length DAF-3 is not observed in wild type or animals lacking TGF- β signaling (Patterson, Kowook et al. 1997). In agreement with other studies we did not observe nuclear DAF-3 when expressing *daf-3(+)* in *daf-1(m40); daf-3(mgDf90)* animals under the *daf-1* or *tdc-1* promoters (Figure 5). However, we did observe some nuclear DAF-3 in select ciliated sensory neurons when expressing *daf-3(+)* in *daf-1(m40); daf-3(mgDf90)* animals under the *osm-6* promoter (Figure 5). This suggests that nuclear accumulation of DAF-3 may occur in cases when interneurons are wild type for TGF- β signaling, while ciliated sensory neurons are mutant for TGF- β signaling. Previous studies demonstrated that a truncated but still functional DAF-3 did localize to the nucleus (Patterson, Kowook et al. 1997); therefore, one possibility for the phenotype observed is that ciliated sensory neurons express a differently spliced form of DAF-3. Whether or not this observation is functional significance remains to be established.

Ablation of RIM and RIC in wild type and *daf-1(m40)* affects feeding rate not fat storage

daf-1 rescue experiments demonstrated that *daf-1* signaling in RIM and RIC is sufficient for the TGF- β pathway to regulate pharyngeal pumping and fat storage. Furthermore, *daf-3* rescue experiments described above suggest that RIM and RIC may also be sufficient. In order to test this directly we ablated RIM and RIC neurons in *daf-1(m40)* and wild type animals. In *C. elegans* overexpression of the mammalian caspase ICE can result in neuronal ablation (Hills, Brockie et al. 2004). Using this strategy, we

ablated RIM and RIC by expressing ICE under the *tdc-1* promoter. Either wild type or *daf-1(m40)* animals were injected with *ptdc-1::ICE*, to ablate RIM and RIC, and *ptdc-1::gfp*, in order to determine successful ablation by loss of GFP signal in RIM and RIC. Ablation of RIM and RIC in wild type animals did not affect fat storage but led to a slight reduction in pharyngeal pumping rate (Figure 6A-C). Ablation of RIM and RIC in *daf-1(m40)* slightly increased the pharyngeal pumping rate of *daf-1(m40)* animals but did not affect fat storage (Figure 6A-C). These data suggest that RIM and RIC are sufficient for TGF- β signaling to regulate pharyngeal pumping, but not fat storage.

We also attempted to create a dominant negative *daf-1* receptor to determine if TGF- β signaling in RIM and RIC is sufficient to regulate pharyngeal pumping and fat storage. In both mammals and *Drosophila*, truncation of DAF-1 results in a dominant negative protein that can bind ligand but cannot signal to downstream SMADs (Brand and Schneider 1995; Haerry, Khalsa et al. 1998). We truncated *daf-1* after amino acid 300 (*daf-1 Δ 300*), 241 (*daf-1 Δ 241*), and 200 (*daf-1 Δ 200*) to attempt to create a dominant negative. Transgenic animals expressing the *daf-1 Δ 241* and *daf-1 Δ 200* receptors under the *daf-1* promoter did exhibit some *daf-1(m40)* phenotypes including dauer formation and increased fat stores; however, these phenotypes were not suppressed by the co-SMAD *daf-3*. Thus, the observed phenotypes are not due to a disruption of the TGF- β pathway, but may be due to aberrant changes in the plasma membranes of cells expressing the construct. We observed membrane blebbing and irregular intracellular vesicles expressing GFP in transgenic animals expressing *pdaf-1daf-1 Δ 241::gfp* and *pdaf-1::daf-1 Δ 200::gfp* (data not shown). We did not observe any phenotypes associated with expression of the *daf-1 Δ 300* receptor under the *daf-1* promoter.

Discussion

TGF- β signaling is sufficient in two interneurons to regulate feeding behavior, fat storage, and dauer formation

The neuron specific rescue experiments described here reveal that DAF-1 signaling in two interneurons is sufficient to rescue the pharyngeal pumping, fat storage, and dauer phenotypes of *daf-1(m40)* animals. Restoration of DAF-1 signaling in large sets of ciliated neurons, or in interneurons not including RIM or RIC, failed to rescue mutant phenotypes of *daf-1(m40)*. RIM and RIC are both interneurons that project into the nerve ring or central nervous system of *C. elegans* (White, Southgate et al. 1986). In addition, RIM can function as a motor neuron regulating head movement when animals are moving backwards (Alkema, Hunter-Ensor et al. 2005). As interneurons, these cells integrate signals from other primary sensory cells and interneurons; thus, it is possible that other inputs could be integrated with TGF- β signaling to determine fat storage and feeding rates (White, Southgate et al. 1986). Interestingly, RIM and RIC do not receive any synapses from ASI, where the TGF- β ligand DAF-7 is secreted. The DAF-1 receptor on RIM and RIC must therefore receive the DAF-7 signal in an extrasynaptic manner. This non-synaptic communication is observed for other peptides such as leptin, which travel great distances before binding receptors in the hypothalamus and other brain regions.

TGF- β signaling may be necessary in two interneurons to regulate feeding behavior, fat storage, and dauer formation

While expression of *daf-1(+)* in RIM and RIC is sufficient to rescue *daf-1* mutant phenotypes, there is a possibility that other unidentified neurons may also be important.

In order to determine if TGF- β signaling in RIM and RIC is necessary for feeding and fat storage, we took several approaches to create animals with wild type signaling in all other cells but RIM or RIC. In the first approach, we expressed the co-SMAD *daf-3* in *daf-1(m40); daf-3(mgDf90)* animals. DAF-3 is inhibited by active TGF- β signaling and suppresses the *daf-1(m40)* phenotypes. Cells expressing *daf-3* in the *daf-1(m40); daf-3(mgDf90)* animals will be mutant for *daf-1* while neurons not expressing *daf-3* will be wild type. In this experiment, we found that expression of *daf-3* in RIM and RIC is necessary to uncover the pharyngeal pumping and fat storage phenotypes of *daf-1(m40)* animals. This suggests that TGF- β signaling is necessary in RIM and RIC.

In contrast, loss of RIM and RIC does not suppress the fat storage phenotype of *daf-1(m40)* or cause fat accumulation in wild type animals. Ablation of RIM and RIC shows that these cells are necessary for pharyngeal pumping, since ablation of RIM and RIC in wild type or *daf-1(m40)* animals results in animals that pump at the same rate. In addition, it suggests that RIM and RIC may be capable of both positive and negative regulation on pharyngeal pumping since either phenotype can occur depending on the genetic background of the animal. Other attempts to address this question using a dominant negative were not successful. In *C. elegans* the TGF- β receptor DAF-4 is expressed in both a full-length and truncated secreted form, which is proposed to antagonize TGF- β signaling (Gunther and Riddle 2004). Overexpression of such a protein could be one additional way to attempt to create a dominant negative.

There are several reasons why we observe conflicting results in ablation studies to examine whether TGF- β signaling in RIM and RIC is necessary for regulation of fat storage and feeding behavior. First, ablating the entire neuron surely disrupts many more

signaling pathways than just the TGF- β pathway. For example, if RIM and RIC release factors that both promote and inhibit fat storage ablating the neurons may not produce a fat phenotype even though these neurons are important in energy homeostasis. In addition, ablation of RIM and RIC could result in compensatory signaling to make up for their absence. In fact, researchers observed a similar phenomenon when attempting to ablate NPY/ AgRP neurons, which normally function to promote feeding and fat storage. In mice, ablation of NPY and AgRP neurons in neonatal mice did not alter feeding or energy balance in these animals (Luquet, Perez et al. 2005). Conversely, rapid ablation of these neurons in adult animals led to severe anorexia (Gropp, Shanabrough et al. 2005; Luquet, Perez et al. 2005). Using the ICE caspase we ablated RIM and RIC in embryos or young L1 larval animals; however, studies in mice suggest that homeostatic compensation may occur when neurons regulating energy balance are lost early in development (Morton, Cummings et al. 2006). These studies also indicate that we may obtain different results if we ablated these neurons in older animals.

DAF-1 and DAF-3 signaling in RIM and RIC are sufficient for regulation of energy homeostasis, feeding, and dauer formation. In addition, many other *C. elegans* neurons do not play a similar role. Here we define a very specific neural circuit for TGF- β regulation of feeding behavior and fat storage. We demonstrate that this neural network is not distributed, but that TGF- β signaling in RIM and RIC regulates both feeding behavior and fat storage. Identifying the signals emanating from RIM and RIC to regulate feeding behavior and fat storage will help us understand the role of TGF- β signaling in energy homeostasis.

Table 1: Expression of *daf-1* in Interneurons Is Sufficient to Rescue the Dauer Formation, Pharyngeal Pumping Rate, and Fat Storage Phenotypes of *daf-1(m40)* Mutants.

	Genetic background	Promoter driving <i>daf-1::gfp</i>	# of Cells expressing <i>daf-1::gfp</i>	% Dauer (n) ^a	% WT pumping rate ^b	Fat content ^c
Control	<i>N2</i> (WT)	none		0	100 ± 2.8*	wild type
	<i>daf-1(m40)</i>	none		98 (480)	76 ± 2.7	increased
	<i>daf-1(m40)</i>	<i>daf-1</i>	many (>80) neurons	2 (179)	97 ± 2.1*	wild type
Pan-Neuronal	<i>daf-1(m40)</i>	<i>egl-3</i>	many (>80) neurons	3 (112)	98.2 ± 3*	wild type
Sensory Neurons	<i>daf-1(m40)</i>	<i>bbs-1</i>	60 ciliated neurons	99 (142)	75 ± 6.7	increased
		<i>osm-6</i>	56 ciliated neurons	100 (140)	69 ± 3.5	increased
		<i>daf-7</i>	2 (ASI)	100 (79)	N/D	increased
Pharynx	<i>daf-1(m40)</i>	<i>glr-8</i>	25 pharyngeal neurons and interneurons	56 (147)	81.7 ± 4	increased
		<i>glr-7</i>	9 pharyngeal neurons	98 (249)	71 ± 6.0	increased
		B0280.7	5 pharyngeal gland	98 (181)	74 ± 5.4	increased
Interneuron	<i>daf-1(m40)</i>	<i>glr-2</i>	24	94 (332)	74 ± 2.3	increased
		<i>unc-47</i>	29	100 (102)	72 ± 4.7	increased
		<i>unc-17</i>	80	37 (174)	76 ± 6.3	increased

<i>glr-5</i>	56	6 (175)	97 ± 1.9*	wild type
<i>glr-4</i>	38	3 (248)	96 ± 2.9*	wild type
<i>glr-1</i>	24	14 (170)	90 ± 4.6*	wild type
<i>ggr-1</i>	10	94(11 9)	77 ± 7.1	increased
<i>flp-1</i>	2	100 (216)	76 ± 2.6	increased
<i>dop-1</i>	12	100 (231)	76 ± 6.1	increased
<i>nmr-2</i>	12	2 (221)	95 ± 3.2*	wild type
<i>tdc-1</i>	4 interneurons and UV1 cells	1 (188)	98 ± 3.1*	wild type
<i>tbh-1</i>	2	57 (178)	84 ± 3.1*	wild type

^a % animals (n=100 to 400) that enter dauer when placed on food plates as L1s at 25°C.

^b Average pumping rate of well-fed, young gravid adults. P-values relative to *daf-1(m40)* pumping rate were determined by ANOVA with Bonferroni post-test. P-values <0.001 were considered significant and, in case of transgenes, indicate rescue of *daf-1(m40)* pumping phenotype.

^c Fat phenotypes were determined by Sudan Black B. staining. In each case transgenic animals were compared to non-transgenic *daf-1* animals fixed and stained in the same tube to minimize staining variation.

Table 2: Egg-Retention Phenotypes of *daf-1(m40)* Transgenics.

	Genetic background	Promoter driving <i>daf-1::gfp</i>	Eggs Retained
<i>Control</i>	<i>N2 (WT)</i>	none	+
	<i>daf-1(m40)</i>	none	+++
	<i>daf-1(m40)</i>	<i>daf-1</i>	+
Pan Neuronal	<i>daf-1(m40)</i>	<i>egl-3</i>	+
Sensory Neuron	<i>daf-1(m40)</i>	<i>bbs-1</i>	+++
		<i>osm-6</i>	+++
		<i>daf-7</i>	+++
Pharyngeal Neuron	<i>daf-1(m40)</i>	<i>glr-8</i>	+++
		<i>glr-7</i>	+++
		<i>B0280.7</i>	+++
Interneuron	<i>daf-1(m40)</i>	<i>glr-2</i>	+++
		<i>unc-47</i>	+++
		<i>unc-17</i>	+
		<i>glr-5</i>	++
		<i>glr-4</i>	++
		<i>glr-1</i>	+++
		<i>ggr-1</i>	+++
		<i>flp-1</i>	+++
		<i>dop-1</i>	++
		<i>nmr-2</i>	++
		<i>tdc-1</i>	++
		<i>tbh-1</i>	+++

Indicated promoters were used to express *daf-1::gfp* in *daf-1(m40)* strain. Young, well-fed wild type animals retained ~10 eggs on average. This phenotype is denoted as (+). *daf-1(m40)* mutants retained ~24 eggs, denoted as (+++). An intermediate egg-retention phenotype is denoted as (++) . Phenotypic examples are shown and quantitated in Figure 2.

Table 3: Expression Patterns of Interneuron-specific Promoters

Promoter Driving <i>daf-1::gfp</i>	Cells Expressing <i>daf-1::gfp</i>	Rescue of <i>daf-1(m40)</i> phenotypes^a
<i>glr-2</i>	AVA, AVD, AVE, PVC, RMD, AIA, AIB, AVG, RIG, RIA, M1, RIR	None
<i>unc-47</i>	19 D ventral cord neurons, RMEs, AVL, RIS, DVB	None
<i>unc-17</i>	IL2, URA, URB, SAA, SAB, SIA, SIB, SMB, SMD, RMD, AIA, M1, M2, M5, VA, VB, VC, DA, DB, SDQ, HSN, ALN, PLN	None
<i>glr-5</i>	AVA, AVB, AVD, AVE, PVC, <u>RIM</u> , <u>RIC</u> , RMD_SMD, SIB, RME, AVK, RMG, SABVL, SABVR, SABD, RIF, VC, LUA, PVQ, URB, URY, URA DVA, AIB, HSN	Full
<i>glr-4</i>	AVA, RMD, SMD, SAA, SIB, RIB, <u>RIM</u> , AVH, FLP, RMG, DVA, AUA, PVD, URY, URA, SAB, RIF, DB, PVU	Full
<i>glr-1</i>	AVA, AVB, AVD, AVE, PVC AIB, RMD, <u>RIM</u> , SMA, AVG, PVQ, URY	Full
<i>ggr-1</i>	AIB, PVR, PVQ, AVH, SMDV	None
<i>flp-1</i>	AVK	None
<i>dop-1</i>	RIS, AVM, ALM, ALN, PLN, PVQ	None
<i>nmr-2</i>	AVA, AVD, AVE, <u>RIM</u> , AVG, PVC	Full
<i>tdc-1</i>	<u>RIM</u> and <u>RIC</u> , UV1 cells	Full
<i>tbh-1</i>	<u>RIC</u>	Partial

daf-1::gfp expression was targeted to indicated cells by each of the listed promoters. Cell names are listed. See www.wormbase.org for additional information.

^a Phenotypes of *daf-1(m40)* mutants are constitutive larval dauer formation, adult excess fat accumulation, reduced pumping rate, and egg-retention. All *daf-1(m40)* phenotypes were rescued by promoters that targeted *daf-1::gfp* to RIM or RIC interneuron (underlined).

Table 4: List of Promoters used to rescue *daf-3* in *daf-1(m40);daf-3(mgDf90)*.

	Genetic background	Promoter	Cells expressing <i>daf-3::gfp</i>	% Dauer (n) ^a	% WT pumping rate ^b	Fat Phenotype ^c
Control	N2 (WT)	none		0	100 ± 3.4	wild type
	<i>daf-1(m40)</i>	none		98 (278)	81 ± 4.8 p<0.001	increased
	<i>daf-3(mgDf90)</i>	none		0 (234)	98 ± 2.9	
	<i>daf-1(m40); daf-3(mgDf90)</i>	none		0 (187)	97 ± 3.0	wild type
	<i>daf-1(m40); daf-3(mgDf90)</i>	<i>daf-1</i>	many (>80) neurons	40 (181)	83 ± 4.0 p<0.001	
Sensory Neurons	<i>daf-1(m40); daf-3(mgDf90)</i>	<i>osm-6</i>	56 ciliated neurons	0 (125)	97 ± 4.6	wild type
Pharyngeal Neurons	<i>daf-1(m40); daf-3(mgDf90)</i>	<i>glr-7</i>	9 pharyngeal neurons	0 (98)	95 ± 5.7	wild type
Specific Inter-neurons	<i>daf-1(m40); daf-3(mgDf90)</i>	<i>tdc-1</i>	4	18 (186)	84.5 p<0.001	increased
		<i>tbh-1</i>	2	0 (139)	92 ± 5.7	wild type

^a % animals (n=100 to 400) that enter dauer when placed on food plates as L1s at 25°C.

^b Average pumping rate of well-fed, young gravid adults. P-values relative to wild type (N2) pumping rate were determined by ANOVA with Bonferroni post-test. P-values <0.001 were considered significant.

^c Fat phenotypes were determined by Sudan Black B. staining. Comparisons are reported relative to wild type. Quantitations are shown in Figure 4.

Figure 1: Rescue of *daf-1* in RIM and RIC interneurons rescues dauer, pharyngeal pumping rate, and fat storage phenotypes of *daf-1(m40)* mutants.

(A) Examples of neuronal expression patterns of promoters used to express *daf-1::gfp*. Axis indicates P, posterior, A, anterior, D, dorsal, V, ventral positions of animals shown.

(B-C) Examples of tissue selective reconstitution of *daf-1* on pharyngeal pumping rate and fat content. Expression of *daf-1(+)* under its own promoter or *egl-3* pan-neuronal promoter rescued the reduced pharyngeal pumping rate and high fat phenotypes of *daf-1(m40)* animals. Expression of the same *daf-1(+)* fusion under promoters that target ciliated sensory neurons (*osm-6*) or pharyngeal neurons (*glr-7*) failed to rescue any of the *daf-1(m40)* phenotypes. Rescues were obtained using *glr-5* and *tdc-1* promoters that target a limited set of interneurons including RIM and RIC. Asterisks indicate statistical significance relative to *daf-1(m40)* ($p < 0.001$ determined by ANOVA with Bonferroni post-test). Standard deviation bars are shown.

(D) *pdaf-1::daf-1::gfp* co-localized with *ptdc-1::RFP* which expresses in RIM and RIC interneurons (white arrowheads).

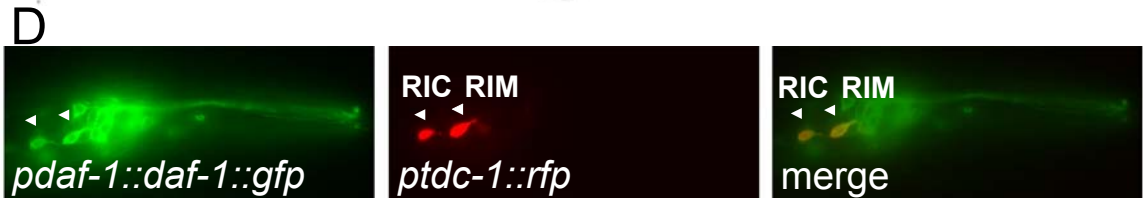
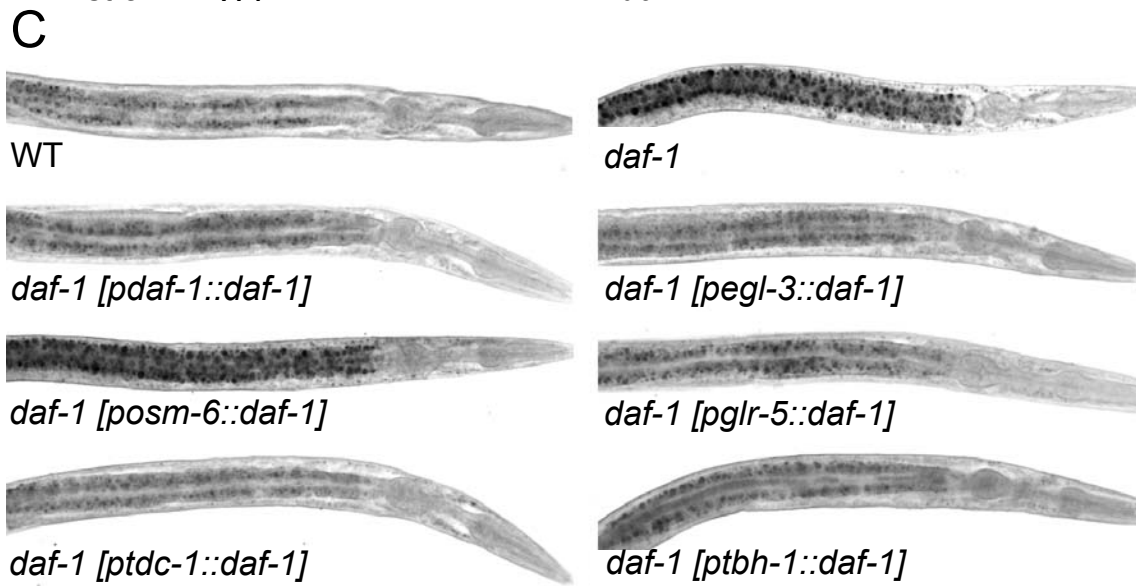
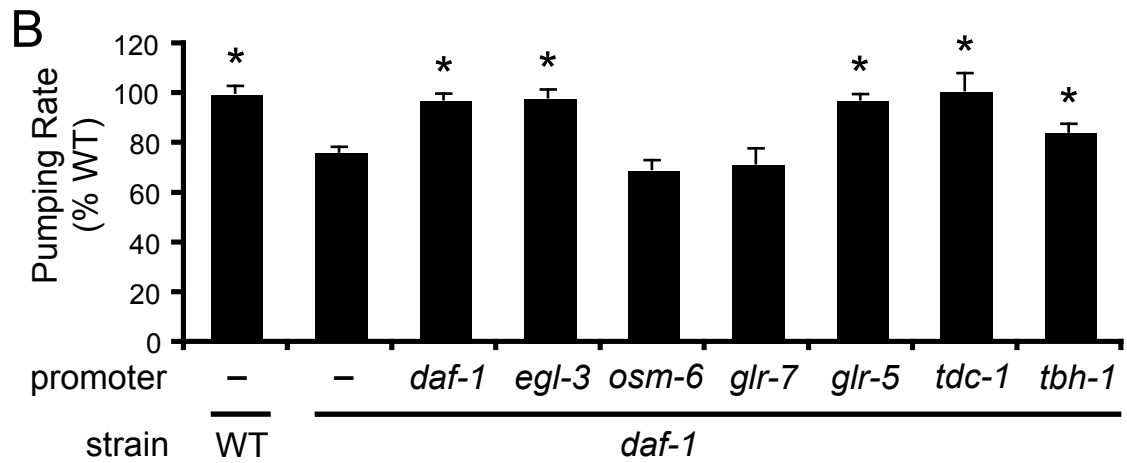
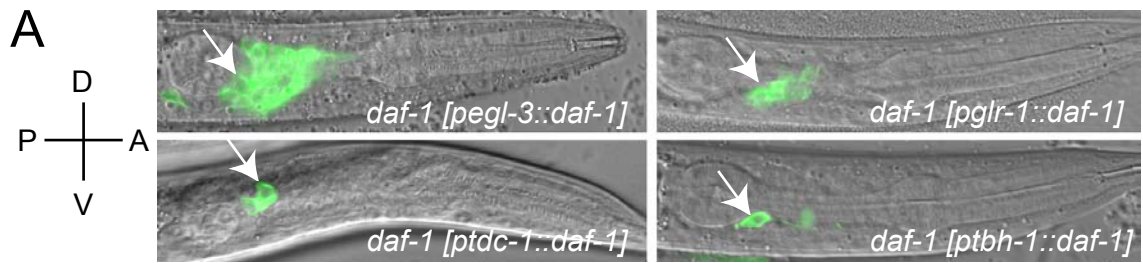
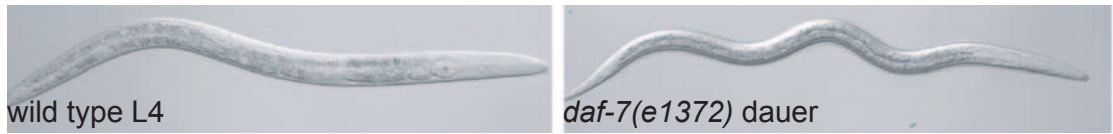


Figure 2: Example of dauer and egg-retention phenotypes

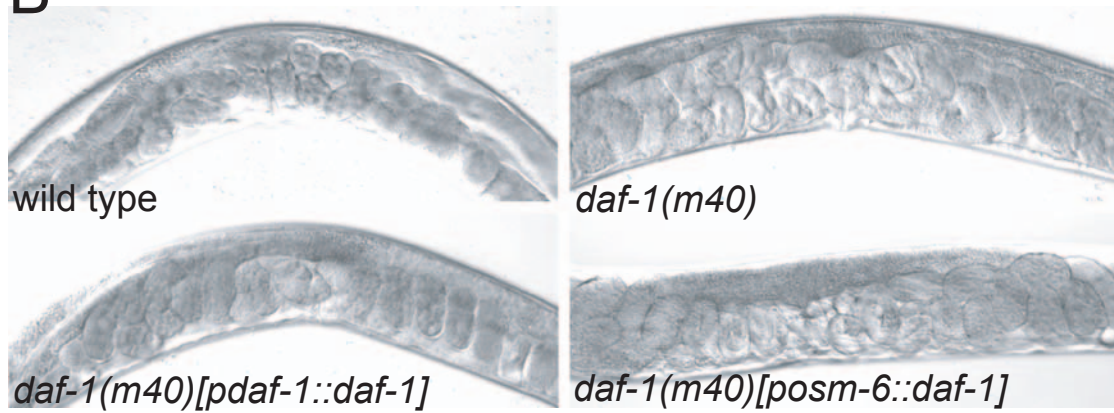
A) Example of wild type L4 and *daf-7(e1372)* dauer animals. Dauers were identified by their characteristic thin morphology and constricted pharynxes.

(B-C) Examples and quantitations of egg-retention in *daf-1(m40)* animals. *daf-1(-)* animals retained more eggs than wild type. This phenotype was rescued when *daf-1(+)* was expressed by the endogenous *daf-1* promoter but not by the *osm-6* sensory neuron promoter. Asterisks indicate statistically significant ($p < 0.001$) from *daf-1(m40)* as determined by ANOVA with Bonferroni post-test. Standard deviation bars are shown.

A



B



C

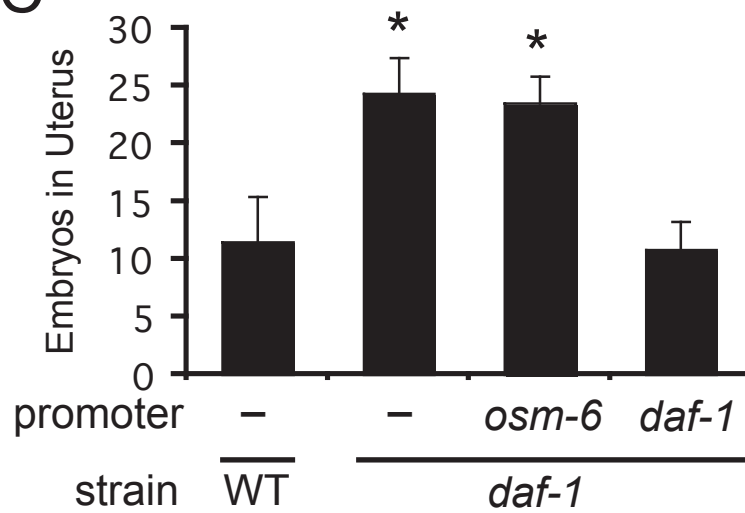


Figure 3: Sudan Black B staining intensity in transgenic animals

Quantitations of relative amounts of Sudan Black B staining intensities for strains shown in Figure 1C. Asterisks indicate statistical significance ($p < 0.001$) as determined by t-test when comparing transgenic animals and co-stained *daf-1(m40)* non-transgenic animals. Standard error bars are shown.

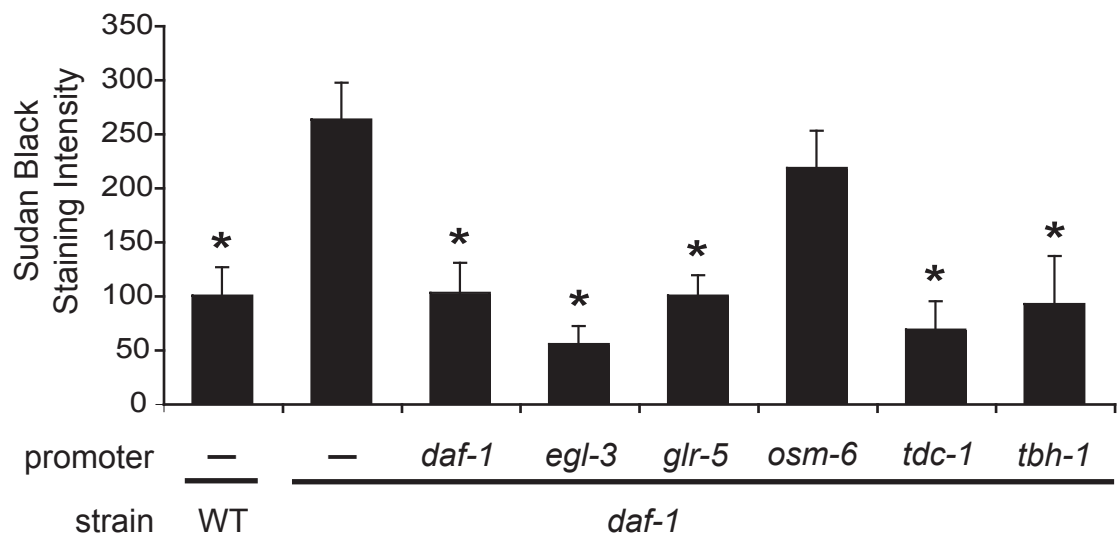


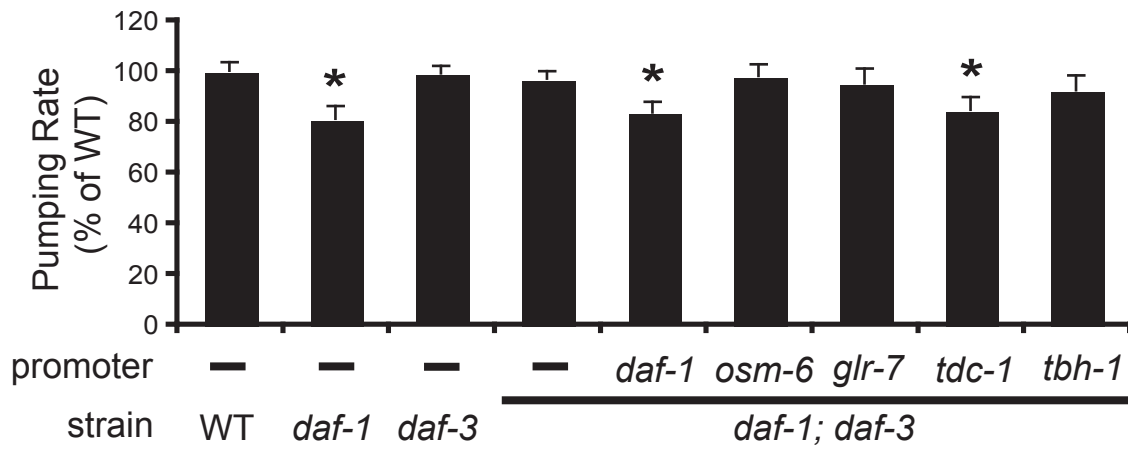
Figure 4: Selective activation of *daf-3* in RIM and RIC interneurons causes fat accumulation and pharyngeal pumping reduction

(A) pharyngeal pumping rate of *daf-1(m40); daf-3(mgDf90)* is reduced by expressing *daf-3(+)* in all neurons or selectively in RIM and RIC using the *tdc-1* promoter.

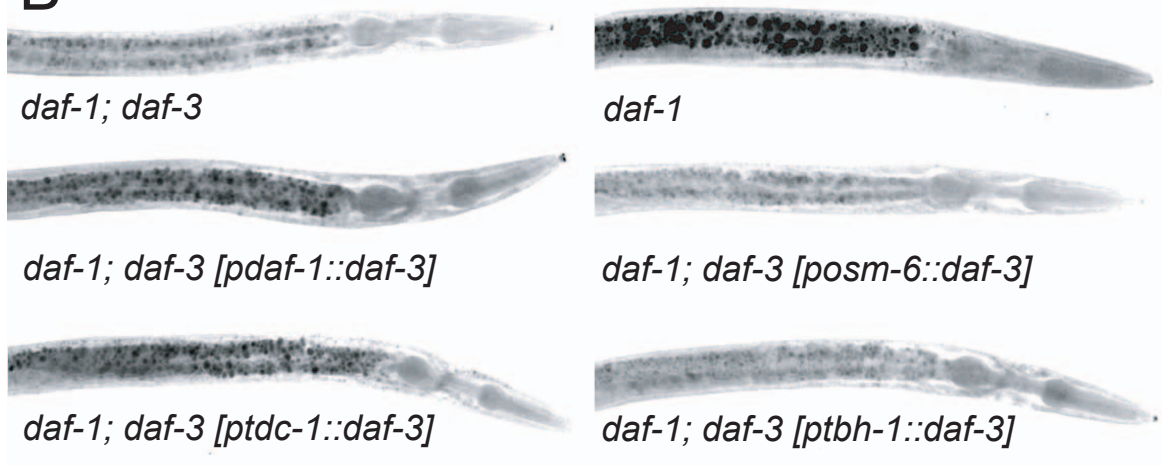
Asterisks indicate statistical significance relative to wild type ($p < 0.001$ determined by ANOVA with Bonferroni post-test). Standard deviation bars are shown.

(B-C) Fat content of *daf-1(m40); daf-3(mgDf90)* animals in which *daf-3* was selectively targeted to subsets of neurons using the indicated promoters. Asterisks indicate statistical significance ($p < 0.001$) as determined by t-test when comparing transgenic animals and co-stained *daf-1(m40); daf-3(mgDf90)* non-transgenic animals. Standard error bars are shown.

A



B



C

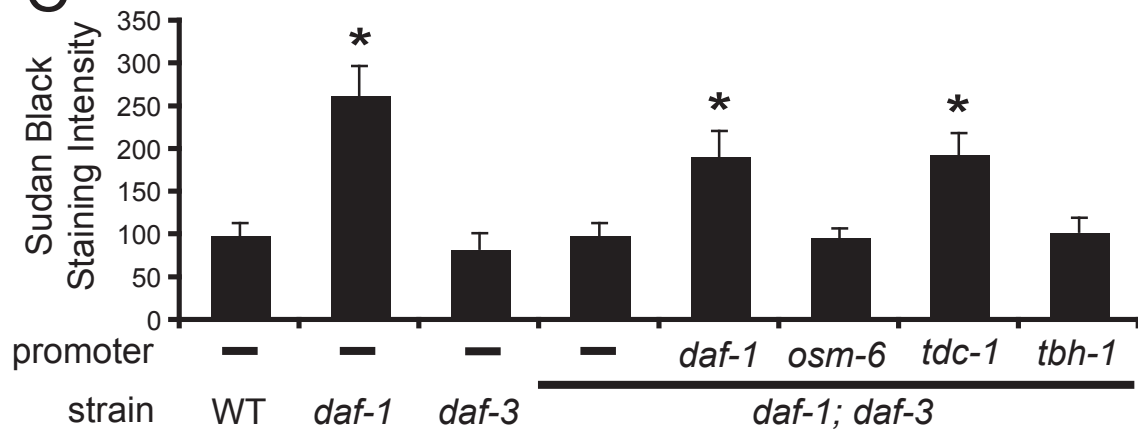


Figure 5: Examples of DAF-3 subcellular localization

Expression of *daf-3(+)* in *daf-1(m40); daf-3(mgDf90)* animals under the *daf-1* and *tdc-1* promoter did not lead to nuclear accumulation of DAF-3 (white arrowheads). However expression with the *osm-6* promoter in sensory neurons led to the accumulation of DAF-3 in the nuclei of some neurons. White arrow indicates nuclear expression of DAF-3 in ASI, ASK, and ADL neurons (from left to right).

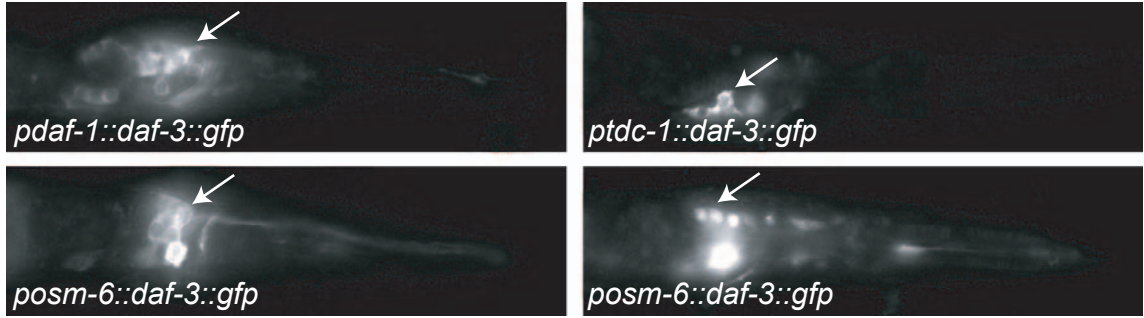


Figure 6: Ablation of RIM and RIC in wild type and *daf-1(m40)*

(A) Ablation of RIM and RIC in wild type using the *ptdc-1::ICE* construct results in a reduced pharyngeal pumping phenotype (90 ± 4.0 % of WT). *daf-1* mutants result in a decreased pharyngeal pumping rate (82 ± 4.0 % of WT) which is increased when RIM and RIC are ablated (89 ± 3.2 % of WT). Standard deviation bars are indicated. **: $p < .001$, * : $p < .01$, ns: $p > .05$ as determined by ANOVA with Bonferroni post-test.

(B-C) Ablation of RIM and RIC using the *ptdc-1::ICE* construct does not alter fat storage of wild type or *daf-1* mutants.

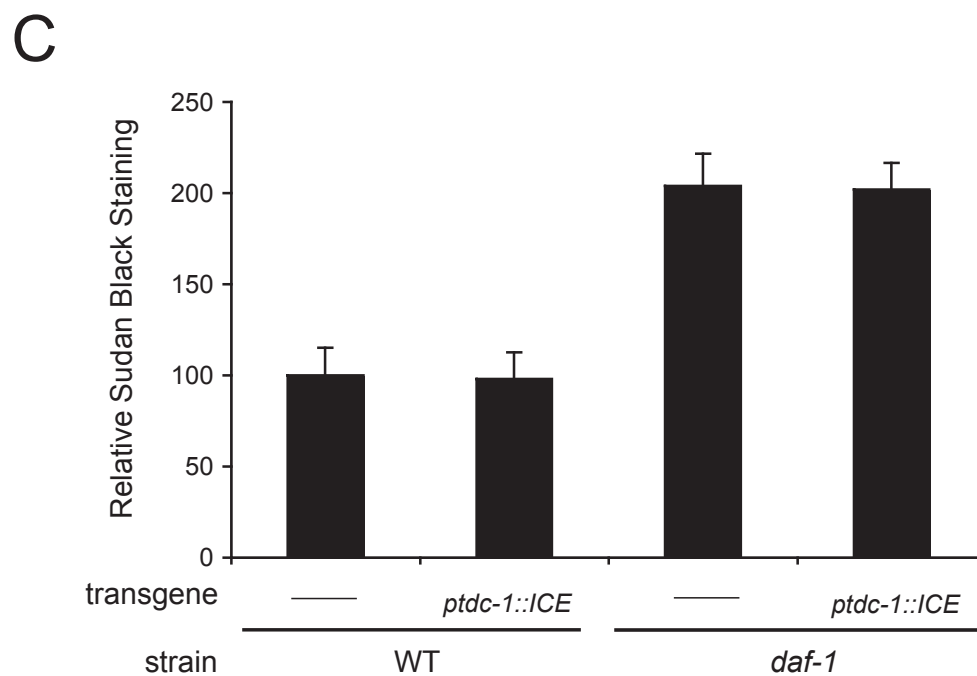
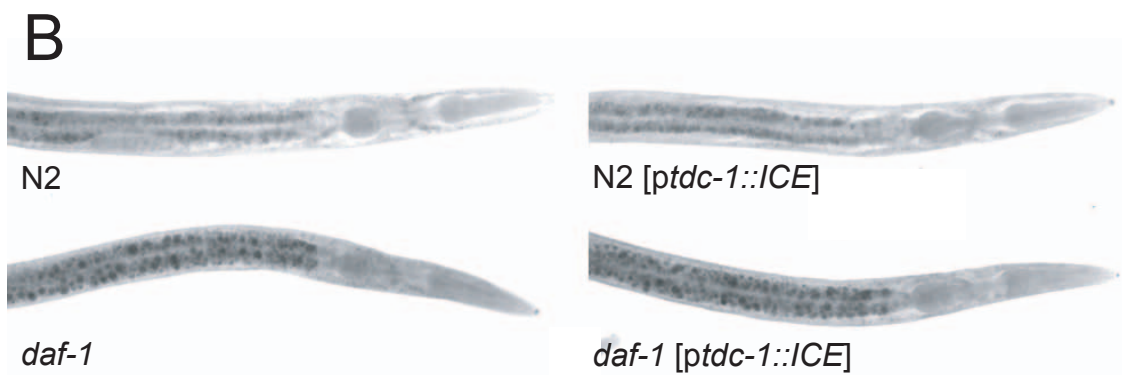
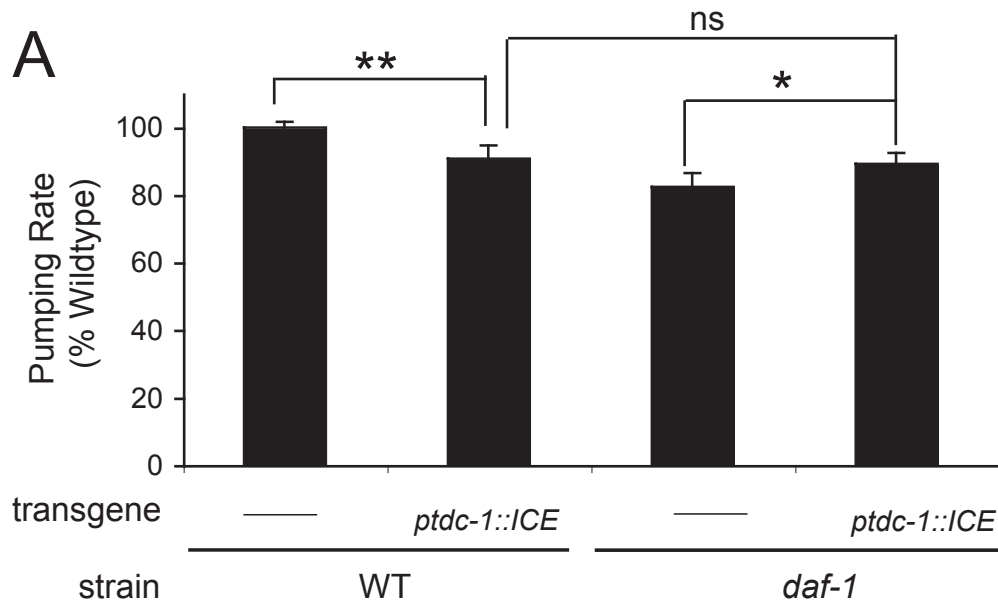
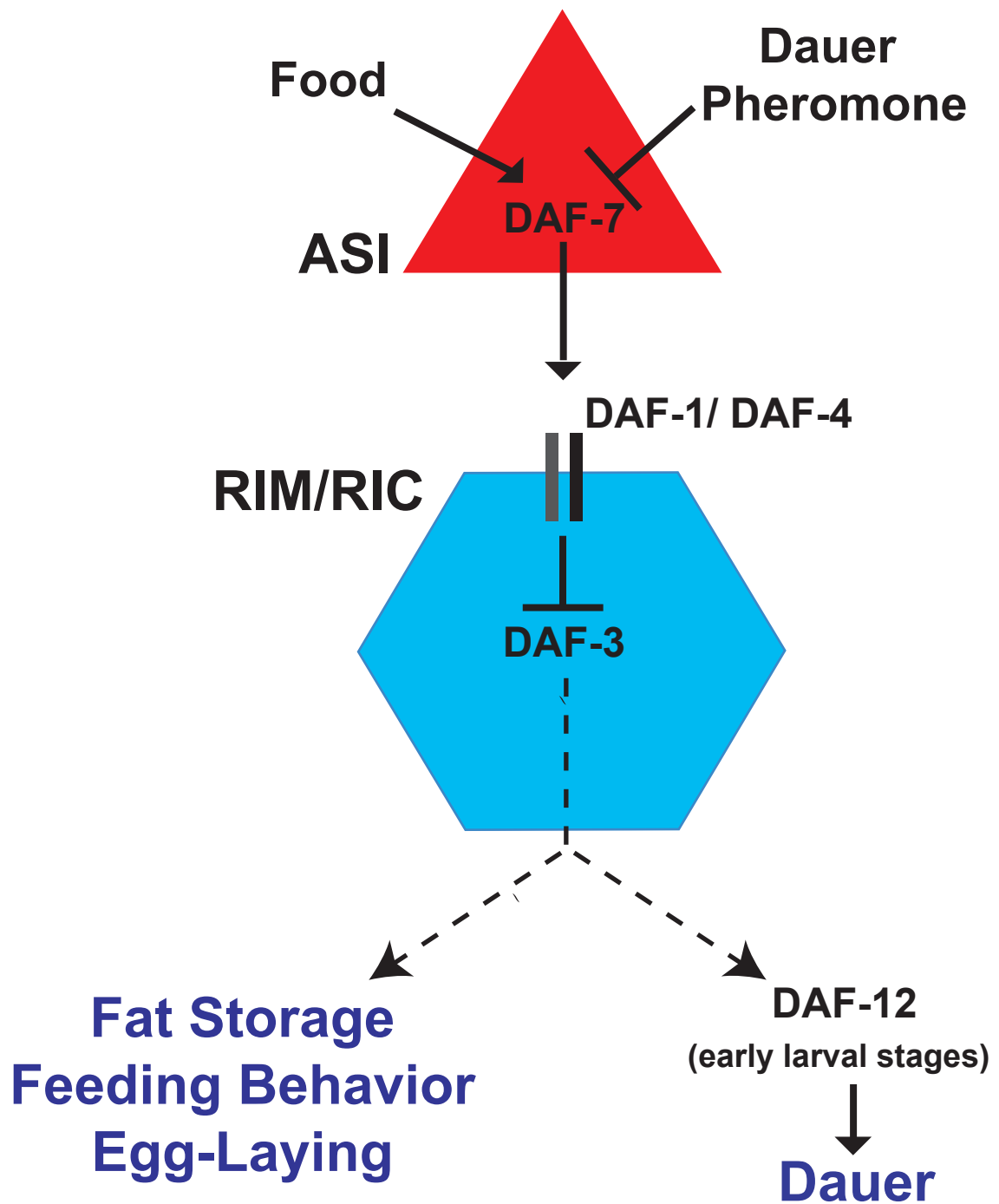


Figure 7: Neurocircuit for TGF- β regulation of feeding behavior, fat storage, and dauer formation

The *daf-7*/TGF- β ligand is released from the ASI sensory neuron in response to food and population density (dauer pheromone concentration). DAF-7 binds to TGF- β receptors DAF-1 and DAF-4 on the ring interneurons RIM and RIC. Signaling outputs from RIM and RIC regulate dauer formation through DAF-12 while feeding behavior, fat storage, and egg-laying are regulated by an independent pathway. Dotted lines indicate unknown signal emanating from RIM and RIC.



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Chapter IV: TGF- β signaling regulates feeding rate through invertebrate neurotransmitters tyramine and octopamine

Introduction

We identified two interneurons key for the regulation of dauer formation, metabolism, and feeding by the TGF- β pathway. These two interneurons, RIM and RIC, do not synapse onto the intestine, the site of fat storage, or the pharynx, suggesting that they must elicit these responses by releasing an effector hormone or neurotransmitter. Tyramine and octopamine, two invertebrate neurotransmitters, are synthesized in RIM and RIC. RIM and RIC express tyrosine decarboxylase (*tdc-1*), which produces the invertebrate neurotransmitter tyramine (Alkema, Hunter-Ensor et al. 2005). Tyramine is then converted to octopamine by the enzyme tyramine β -hydroxylase (*tbh-1*), which is expressed in RIC (Alkema, Hunter-Ensor et al. 2005). Consequently, RIM is a tyraminergetic interneuron, while RIC has the potential to be both a tyraminergetic and octopaminergic interneuron (Alkema, Hunter-Ensor et al. 2005). Mutations in *tdc-1* lead to a loss of both tyramine and octopamine, while mutations in *tbh-1* result in a loss of octopamine and an increase in tyramine (Alkema, Hunter-Ensor et al. 2005).

In *C. elegans*, tyramine and octopamine affect egg-laying rate, pharyngeal pumping, and head movements. Exogenous addition of tyramine or octopamine decreases egg-laying rate; however, only *tdc-1* mutants lacking both tyramine and octopamine show increased egg-laying on bacteria (Horvitz, Chalfie et al. 1982; Alkema, Hunter-Ensor et al. 2005). Tyramine from RIM regulates head oscillations when animals are moving backwards after anterior touch (Alkema, Hunter-Ensor et al. 2005). Addition of octopamine inhibits pharyngeal pumping, or feeding behavior, and inhibits firing of pharyngeal muscle (Horvitz, Chalfie et al. 1982; Rogers, Franks et al. 2001). The direct

effects of tyramine on pumping have not been assayed; however, tyramine abrogates the increase in pharyngeal pumping produced by serotonin (Rex, Molitor et al. 2004). These results imply that, like octopamine, tyramine inhibits pharyngeal pumping. In addition, both *tdc-1* and *tbh-1* mutants pump at a faster rate in the absence of food than wild type animals (Alkema, Hunter-Ensor et al. 2005).

Because octopamine, and possibly tyramine, inhibits pharyngeal pumping, TGF- β signaling could regulate pharyngeal pumping by increasing the release of tyramine or octopamine. Here we show that loss of tyramine or octopamine partially suppresses the pharyngeal pumping phenotype of *daf-1(m40)* animals. We also find that the tyramine GPCR *ser-2* functions in pharyngeal neurons to regulate pharyngeal pumping downstream of the TGF- β pathway.

Results

Loss of tyramine and octopamine suppress the feeding phenotype of animals mutant in *daf-1*

Animals lacking TGF- β signaling have a reduced pharyngeal pumping rate. Similarly, we find that exogenous addition of 4mM tyramine or octopamine reduces the pharyngeal pumping rate of wild type animals (Figure 1A). These results suggest that DAF-1 could inhibit tyramine and/or octopamine release from RIM and RIC to regulate feeding rate. If DAF-1 acts in this manner, *tdc-1* mutants, which lack tyramine and octopamine, or *tbh-1* mutants, which lack only octopamine, should suppress the pharyngeal pumping phenotype of *daf-1(m40)* animals.

We find that both *tbh-1* and *tdc-1* mutations partially suppress the feeding phenotype of *daf-1(m40)* and *daf-7(e1372)* (Figure 1A and data not shown). Both *tbh-1*

and *tdc-1* mutants lack octopamine, suggesting that active TGF- β signaling inhibits octopamine to regulate pharyngeal pumping. However, we previously discovered that expression of *daf-1* in RIM alone was sufficient to rescue the pharyngeal pumping phenotype of *daf-1* mutants (see Chapter III). RIM does not synthesize octopamine or make synaptic connections to RIC; thus, it is likely that tyramine also plays a role in regulating pharyngeal pumping (Figure 1A).

We examined several tyramine G-protein coupled receptors (GPCR's) to determine if tyramine also regulates pharyngeal pumping in the TGF- β pathway. Two tyramine receptors, *ser-2* and *tyra-2*, have been characterized in *C. elegans* (Rex, Molitor et al. 2004; Rex, Hapiak et al. 2005). We find that mutations in the tyramine receptor *ser-2*, but not *tyra-2*, partially suppress the pharyngeal pumping defects of *daf-1(m40)* animals (Figure 1A and Figure 2A). Previous studies have demonstrated that *ser-2* mediates tyramine-dependent pharyngeal pumping phenotypes (Rex, Molitor et al. 2004). *C. elegans* expresses one identified octopamine receptor, *ser-3* (Carre-Pierrat, Baillie et al. 2006; Suo, Kimura et al. 2006); however, mutants in *ser-3* have reduced pharyngeal pumping, preventing us from performing epistasis experiments with *daf-1(m40)* (Figure 2A).

In Chapter II, we showed that dauer pheromone functions through the TGF- β pathway to downregulate pharyngeal pumping in adults. Here we show that mutations in *ser-2* and *tdc-1* suppress the effects of pheromone on pharyngeal pumping (Figure 2B). We also observed that *tdc-1* and *ser-2* mutants fail to downregulate pharyngeal pumping when removed from food (Figure 2B). Although it has been reported that *tbh-1* mutants

also exhibit this phenotype (Alkema, Hunter-Ensor et al. 2005), we did not observe this in our experiments (Figure 2B).

Tyramine GPCR, SER-2, functions in pharyngeal neurons to regulate feeding rate

SER-2 is expressed in about 10% of all *C. elegans* head neurons, including many interneurons and pharyngeal neurons, as well as pharyngeal and head muscle (Tsalik, Niacaris et al. 2003; Rex, Molitor et al. 2004). In order to determine the site of *ser-2* function we expressed the *ser-2* open reading frame (ORF) in muscle and neurons in the *daf-1(m40); ser-2(pk1137)* background. Expression of *ser-2(+)* in pharyngeal muscle using the *myo-2* promoter did not decrease pharyngeal pumping rates of *daf-1(m40); ser-2(pk1137)* animals, although it did produce a developmental delay (Figure 3 and data not shown). Broad expression of *ser-2(+)* in neurons using the *egl-3* promoter reduced pharyngeal pumping rates of *daf-1(m40); ser-2(pk1137)* animals (Figure 3). Expression of *ser-2(+)* in pharyngeal neurons also reduced pharyngeal pumping rates of *daf-1(m40); ser-2(pk1137)* (Figure 3). Thus, *ser-2* expression in pharyngeal neurons regulates pharyngeal pumping downstream of TGF- β signaling. The *glr-7* promoter targets *ser-2(+)* expression to I1, I2, I3, and I6 pharyngeal interneurons, M1 pharyngeal neuron, and NSM neurosecretory motoneurons (Brockie, Madsen et al. 2001). However *ser-2* expression has only been reported in NSM, implicating NSM as the site of *ser-2* function (Tsalik, Niacaris et al. 2003; Rex, Molitor et al. 2004).

Tyramine and Octopamine do not regulate fat storage or dauer formation in the TGF- β pathway

Loss of tyramine or octopamine does not suppress the increased fat storage or dauer formation phenotypes of *daf-1(m40)* and *daf-7(e1372)* animals (Figure 1B-C and

data not shown). Furthermore, triple mutants in *daf-1; tdc-1; daf-12* and *daf-1; tbh-1; daf-12* store the same amount of fat as *daf-1* mutants, yet these animals do not enter dauer and have near wild type pharyngeal pumping rates (Figure 1B-C). In addition, the fat phenotype of *daf-1(m40)* is not suppressed by *tyra-2*, *ser-2*, and *ser-3* receptors or a combination of receptors (data not shown). Molecularly, dauer formation and pharyngeal pumping are regulated independently of fat storage.

TGF- β signaling does not alter the transcription of *tdc-1* or *tbh-1*

One mechanism by which TGF- β signaling could decrease tyramine or octopamine levels is regulation of the transcription of the *tdc-1* and *tbh-1* enzymes. We crossed *daf-7(e1372)* into GFP reporter lines for *tdc-1::gfp* and *tbh-1::gfp*, but did not observe any increase in transcription of these enzymes in the *daf-7(e1372)* background (Figure 4A).

The tyramine receptor *tyra-2* is expressed in the ASI pair of sensory neurons (Rex, Hapiak et al. 2005), which release the TGF- β ligand *daf-7*; thus, tyramine signaling could feedback on *daf-7*/TGF- β . Loss of tyramine and octopamine in the *tdc-1* mutant did not alter the expression of *daf-7::gfp* in ASI (Figure 4B).

Discussion

Distinct signals from RIM and RIC regulate feeding behavior and fat storage

Two invertebrate neurotransmitters are synthesized in the RIM and RIC interneurons. RIM is a tyraminergetic interneuron, while RIC is octopaminergic, but could also secrete tyramine. Epistasis analysis revealed that loss of either tyramine or octopamine partially suppresses the pharyngeal pumping or feeding phenotype of animals mutant in *daf-1*. However, loss of these neurotransmitters had no effect on fat storage or

dauer formation in *daf-1* mutants (Figure 5). Animals mutant in *daf-1* have increased fat storage yet reduced feeding. These two phenotypes can be dissociated downstream of receptor signaling, indicating that decreased feeding does not cause increased fat storage. Rather, both phenotypes are part of a program allowing the animal to adapt to changing environmental conditions. One possibility for the divergence in molecular signals regulating fat storage and feeding is the timeframe over which animals alter these two physiologies. Pharyngeal pumping rate decreases immediately when animals are removed from food, and rises upon return to food. Bath application of neurotransmitters can alter the firing rate of *C. elegans* pharynx in just 10-30 seconds; thus, neurotransmitters can be used to rapidly modulate pharyngeal pumping (Avery and Horvitz 1990; Rogers, Franks et al. 2001). In contrast, most transcriptional changes in metabolic genes occur 2-4 hours after starvation (Van Gilst, Hadjivassiliou et al. 2005). Feeding behavior may therefore be modulated more rapidly than metabolism.

At the molecular level, we observe redundancy in the regulation of feeding behavior by tyramine and octopamine. Surprisingly, we find that mutations in *tdc-1*, *tbh-1*, and *ser-2* suppress the *daf-1* mutant phenotype to the same degree. One observation to account for this could be the fact that *ser-3* mutants have reduced pharyngeal pumping, suggesting that octopamine may have both positive and negative effects on pharyngeal pumping. If this is true, animals mutant in *tdc-1* and *tbh-1* will lack both positive and negative signals regulating pharyngeal pumping. This results in a partial suppression of the *daf-1(m40)* phenotype. We predict that full suppression of the feeding phenotype will emerge when both the tyramine receptor *ser-2* and an as of yet unidentified octopamine

receptor are mutated. Ablation studies in Chapter II also support this model, because ablation of RIM and RIC reduces the pharyngeal pumping rate of wild type animals.

Tyramine and octopamine in *C. elegans*

In other invertebrates, such as insects, tyramine and octopamine have opposing roles on many behaviors; however, in *C. elegans* both neurotransmitters regulate feeding behavior and egg-laying in the same manner (Horvitz, Chalfie et al. 1982; Rex, Molitor et al. 2004; Alkema, Hunter-Ensor et al. 2005). In insects, octopamine and tyramine are involved in “flight or fight” responses. In this model, high octopamine signals stress and promotes increased motor activity. Similarly, we find that *C. elegans* in stressful conditions such as low food or high population density downregulate the TGF- β ligand *daf-7*. This will lead to an increase in octopamine or tyramine signaling, which reduces feeding behavior (Figure 5).

Although tyramine and octopamine are invertebrate neurotransmitters, they have homologous functions to the adrenergic systems of vertebrates. Insertion of crystalline noradrenaline in the lateral hypothalamus enhances feeding, while pharmacological inhibition of its synthesis decreases meal size in satiated rats (Ramos, Meguid et al. 2005). Thus, excess noradrenaline signaling mimics perception of hunger just as *C. elegans* tyramine signaling elicits a food intake behavior normally caused by food deprivation.

Tyramine and octopamine regulate metabolism in insects

In insects, octopamine regulates metabolism during the “flight or fight” response, preparing the animals for long-term energy demanding tasks like flying (Roeder, Seifert et al. 2003; Roeder 2005). High levels of octopamine lead to fat utilization by acting

directly on the fat body (the site of fat storage in insects). In addition, octopamine leads to the release of adipokinetic hormone from corpora cardiaca, which also liberates fatty acids from the fat body (Roeder, Seifert et al. 2003; Roeder 2005). While *tdc-1* and *tth-1* did not suppress the fat storage phenotype of animals lacking TGF- β signaling, exogenous addition of 2.5mM tyramine or octopamine increased Nile red staining in wild type animals (Figure 6). It is unclear what this increase in Nile red staining means; however, mutations in *daf-7* and *daf-1* result in the opposite Nile red phenotype (decreased staining). It is possible that addition of tyramine or octopamine will suppress the fat storage phenotype of *daf-7* and *daf-1* mutants. Tyramine and octopamine function in the *C. elegans* TGF- β pathway to regulate feeding behavior. The role of these neurotransmitters in fat storage is not defined here.

Figure 1: Tyramine and octopamine signaling mediate the reduced feeding rate but not the excess fat storage phenotype of *daf-1(m40)* mutants.

((A) Pumping rates animals exposed to 4mM tyramine or octopamine were reduced to 84% and 81% of wild type, respectively. Reduced pumping rate of *daf-1(m40)* was partially suppressed by loss of both tyramine and octopamine in *daf-1(m40); tdc-1(ok914)* double mutants (91% of wild type pumping rate) or by loss of octopamine in *daf-1(m40); tbh-1(ok1196)* (89% of wild type pumping rate). Reduced pumping rate of *daf-1(m40)* was partially suppressed by the tyraminerbic GPCR *ser-2* (88% of wild type pumping rate). Asterisks indicate statistical significance ($p < 0.001$) as determined by ANOVA with Bonferroni post-test for indicated comparisons. Standard deviation bars are shown.

(B-C) *tdc-1(ok914)*, *tbh-1(ok1196)*, and *daf-12(m20)* mutations do not suppress the high fat phenotype of *daf-1(m40)* animals as visualized by Sudan Black B staining. Asterisks indicate statistical significance ($p < 0.001$) as determined by t-test comparing genotype of interest and *daf-1(m40)* animals. Standard error bars are shown.

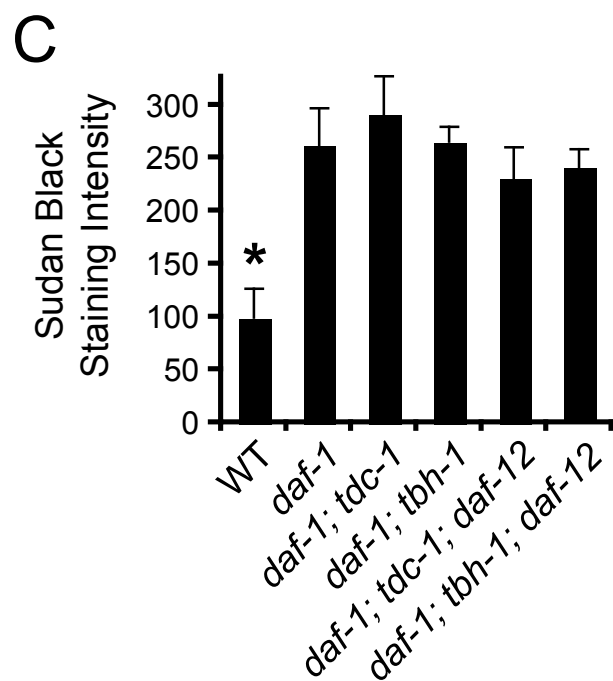
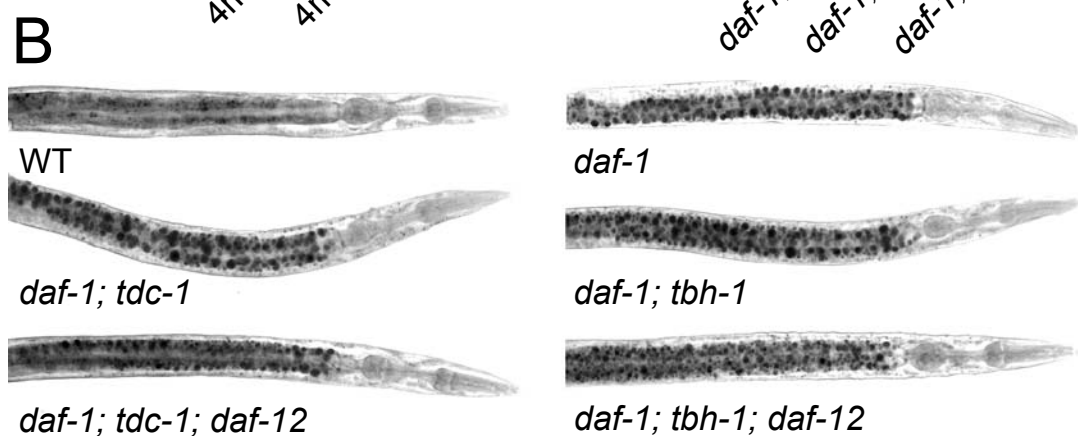
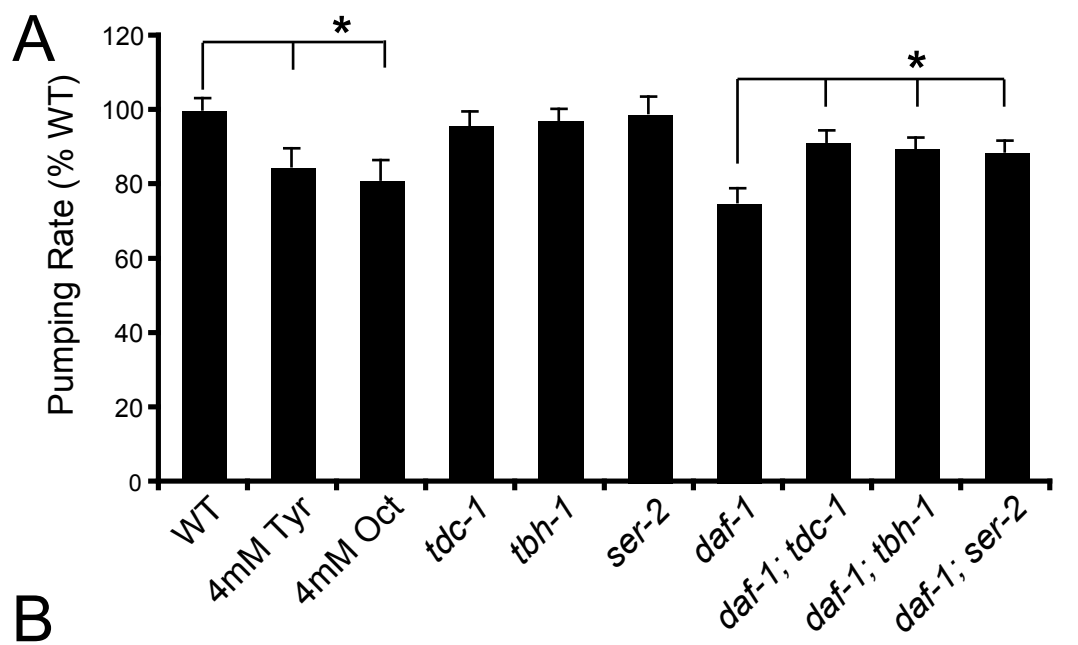


Figure 2: The tyraminerbic GPCR *ser-2* but not *tyra-2* acts downstream of TGF- β signaling to regulate pharyngeal pumping

(A) An inactivating mutation in tyraminerbic GPCR *tyra-2* did not rescue the reduced pumping of *daf-1(m40)*. Pumping rate of *daf-1(m40); ser-2(pk1357); tyra-2(tm1846)* triple mutants was indistinguishable from that of *daf-1(m40); ser-2(pk1357)* (87% of wild type pumping rate). *ser-3* mutations cause a reduction in pharyngeal pumping rate (84% of wild type) Asterisks indicate statistically significant ($p < 0.001$) change from *daf-1(m40)* as determined by ANOVA with Bonferroni post-test. Standard deviation bars are shown.

(B) Removal from food or pheromone treatment caused reduced feeding in wild type. *tdc-1(ok914)* and *ser-2(pk1357)* animals did not respond to pheromone and showed a smaller reduction in feeding relative to wild type when food deprived.

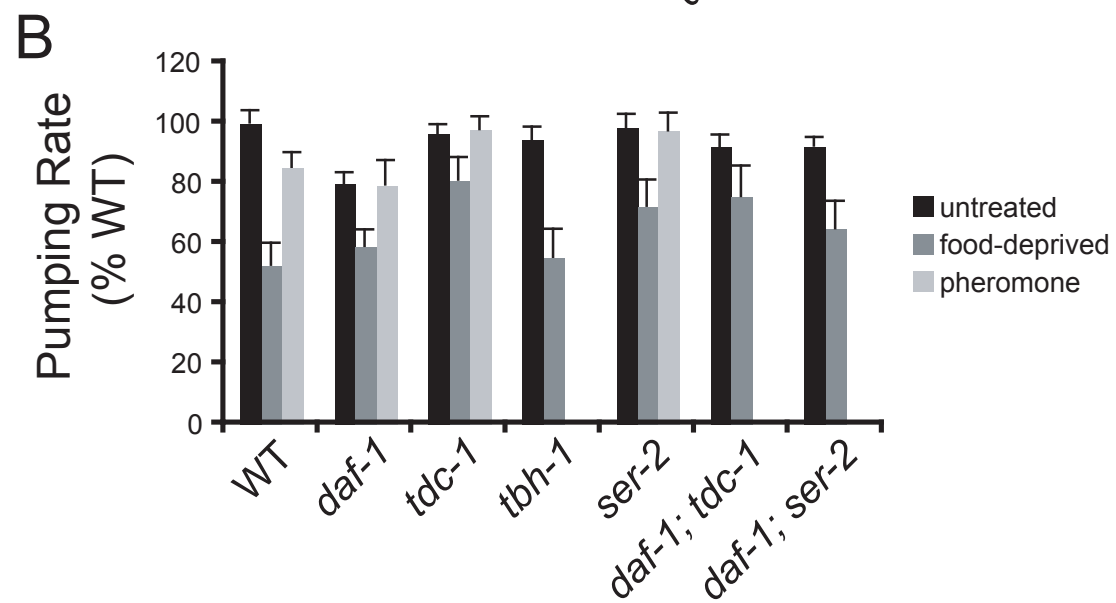
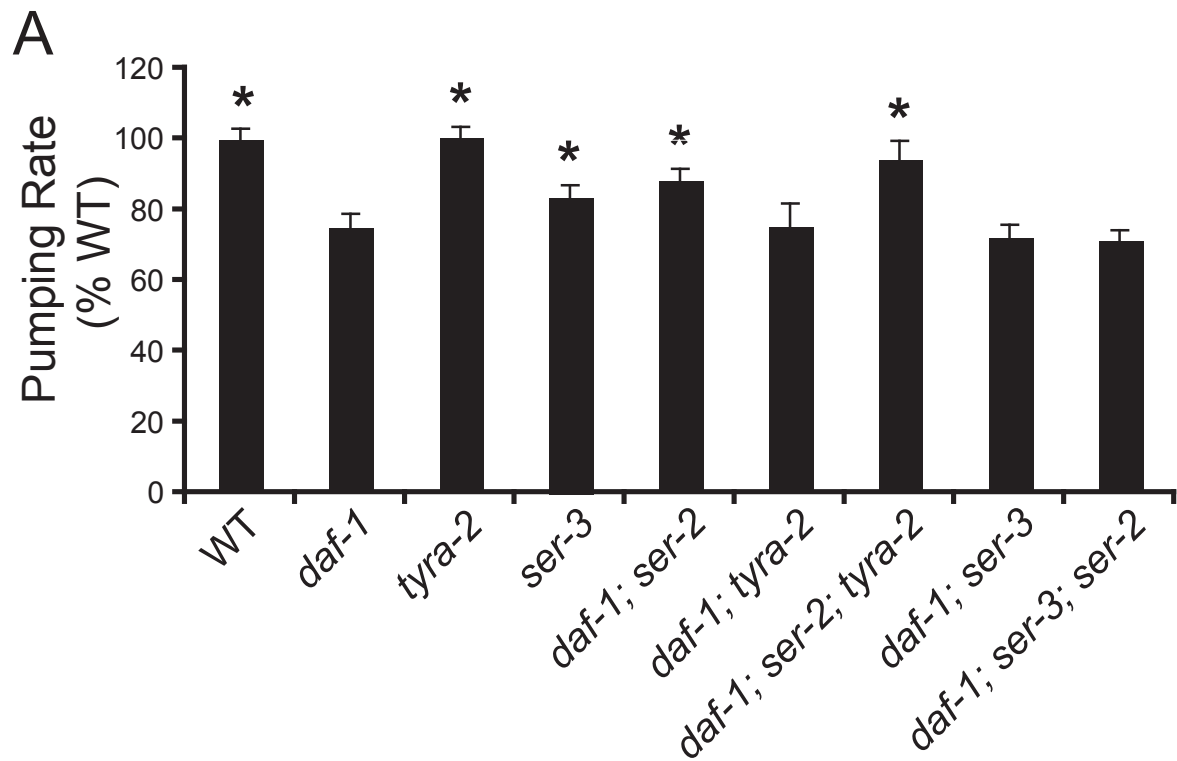


Figure 3: *ser-2* functions in pharyngeal neurons to regulate pharyngeal pumping downstream of TGF- β signaling

Expression of *ser-2*(+) tyraminergetic receptor using tissue specific promoters in *daf-(m40)*; *ser-2(pk1357)* mutants. *myo-2* expresses in pharyngeal muscle, *egl-3* expresses in many neurons, and *glr-7* expresses in pharyngeal neurons. Expression of *ser-2*(+) in a subset of pharyngeal neurons using the *glr-7* promoter is sufficient to allow feeding reduction in *daf-(m40)*; *ser-2(pk1357)* mutants.

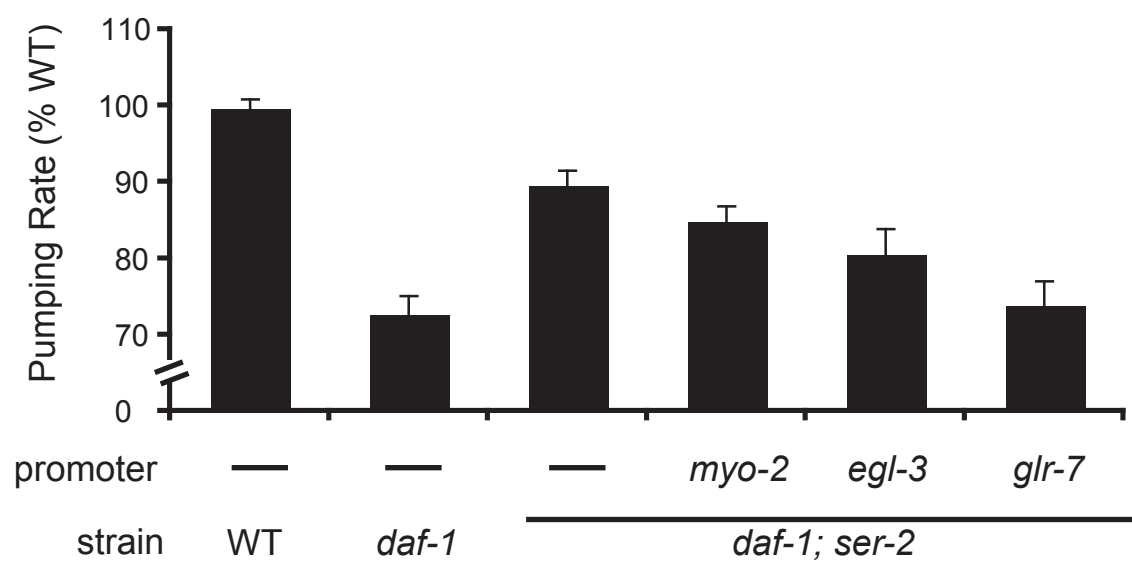
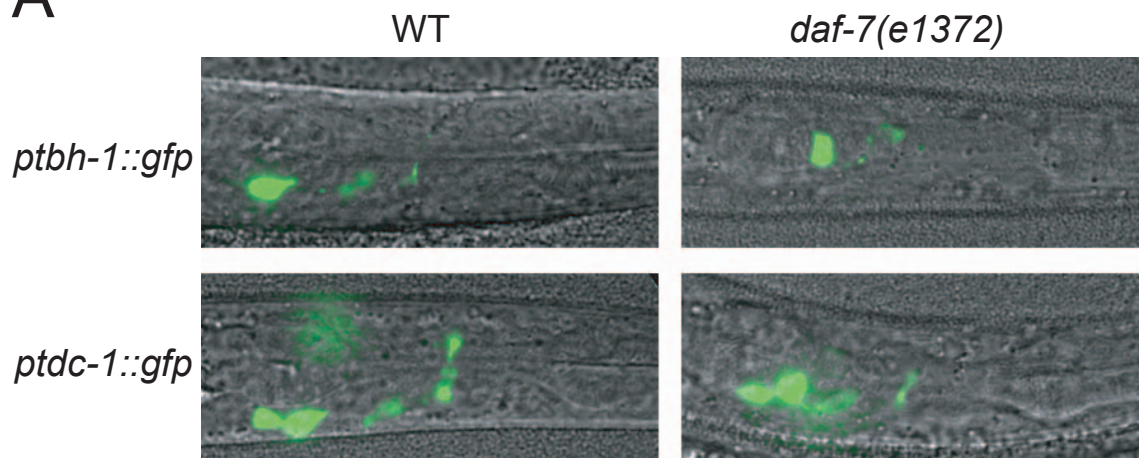


Figure 4: TGF- β signaling does not regulate expression of *ptbh-1::gfp* or *ptdc-1::gfp*

(A) Mutations in *daf-7* do not alter the intensity of either *ptbh-1::gfp* or *ptdc-1::gfp* from wild type.

(B) Loss of tyramine and octopamine in the *tdc-1* mutant does not affect *daf-7::gfp* expression in ASI.

A



B

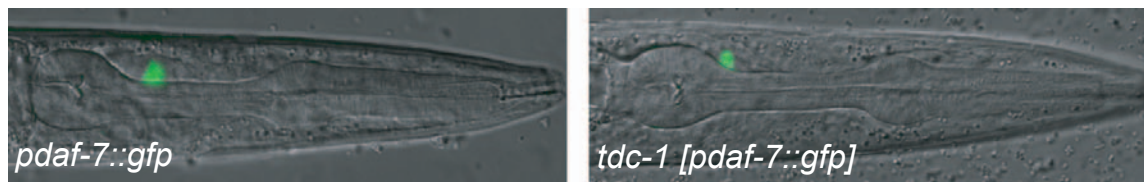


Figure 5: Model for TGF- β regulation of feeding behavior

The *daf-7*/TGF- β ligand is released from the ASI sensory neuron in response to food and population density (increased dauer pheromone concentration). DAF-7 binds to TGF- β receptors DAF-1 and DAF-4 on the ring interneurons RIM and RIC, which are the site of tyramine and octopamine synthesis. Loss of tyramine or octopamine suppresses the feeding phenotypes of *daf-1(m40)* and *daf-7(e1372)* mutants. Specifically, loss of the tyramine receptor SER-2 on pharyngeal neurons regulates feeding behavior downstream of TGF- β ligand and receptor signaling. Despite regulating feeding behavior, tyramine and octopamine do not play a role in fat storage.

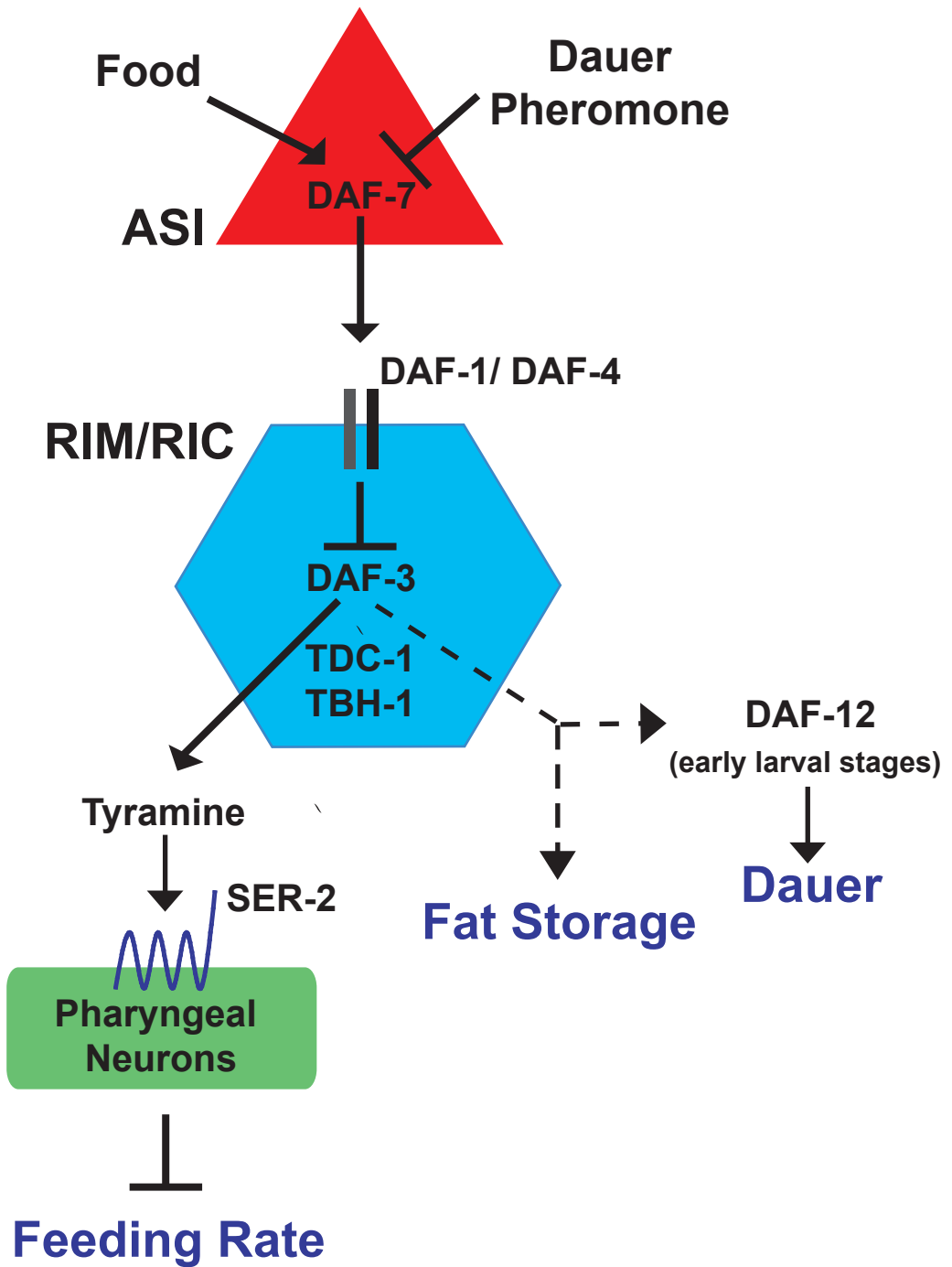
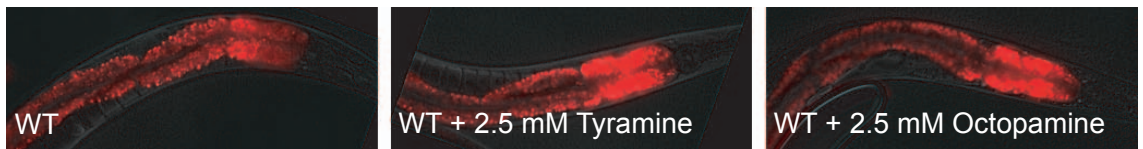


Figure 6: Tyramine and Octopamine increase nile red staining intensity

Exogenous additions of 2.5mM tyramine or 2.5mM octopamine increase the intensity of intestinal nile red staining in wild type animals.



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Chapter V: Genetic suppressors of fat accumulation in TGF- β mutants

Figure 5A contributed by Carissa L. Perez^{1,2} and Marc R. Van Gilst¹

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Introduction

We have identified two interneurons, RIM and RIC, that are important for the regulation of feeding behavior and fat storage in *C. elegans*. Identification of these neurons led us to investigate the role of the invertebrate neurotransmitters that regulate feeding behavior, tyramine and octopamine. We find that mutants lacking tyramine or octopamine partially suppress the feeding defects of TGF- β mutants *daf-1* and *daf-7*, yet do not affect fat accumulation in these animals. In addition, we know that pathways functioning downstream of TGF- β for dauer formation do not affect fat storage. In order to determine molecular pathways downstream of TGF- β signaling that regulate fat storage, we performed a genetic suppressor screen to identify mutations that suppress the fat storage, but not dauer formation phenotype of *daf-1(m40)* animals.

Sudan black staining requires animals to be fixed and cannot be performed on a heterogenic population, making it an unsuitable method to use in a screen. Instead, we took advantage of the reduced Nile red staining phenotype of *daf-1(m40)* animals as a proxy for the Sudan black phenotype. We believed this strategy would identify suppressors of the fat storage phenotype, because mutation in the Co-SMAD, *daf-3*, suppresses both the Nile red and the Sudan black phenotypes of *daf-1(m40)* animals. Similarly, mutation in the nuclear hormone receptor, *daf-12*, fails to suppress either the Nile red or the Sudan black fat phenotype of *daf-1* mutants. From this screen, we expect to identify many alleles of *daf-3* and *daf-5*; however, these alleles will not be studied further since they will suppress both the Nile red and dauer formation phenotypes of *daf-1(m40)* animals (Inoue and Thomas 2000). Using this screening strategy we identified

~30 mutations that suppress the Nile red and dauer formation phenotype of *daf-1(m40)* animals, but only 3 mutations that suppress only the Nile red phenotype. In this screen we find that G-protein signaling through metabotropic glutamate receptors regulates fat storage downstream of TGF- β signaling.

Results

EMS Mutagenesis Screen

We screened for suppression of the low Nile red phenotype in 125,000 F₂ animals from 1,000 mutagenized P₀ *daf-1(m40)* animals (Figure 1). We initially identified 160 potential suppressors, of which 10% were sterile. We transferred each F₂ animal identified as a Nile red suppressor to its own plate, where it laid F₃ progeny (Figure 1). In the first rescreen, we assayed the Nile red phenotype of these mixed stage F₃ animals and determined that 23 % of the originally identified mutants failed to repeat. We then assayed for dauer formation and Sudan black phenotype of mixed stage F₄ animals (Figure 1).

After additional rounds of rescreening using synchronized populations of animals, we identified 30 lines from 15 pools that suppress the dauer formation, Sudan black, and Nile red phenotypes of *daf-1(m40)* animals (Figure 1). Some of these lines are likely to be siblings since they originated from the same F₁ pool, and many are expected to be alleles of *daf-5* since other similarly sized mutagenesis screens isolated 12 alleles of this gene (Inoue and Thomas 2000). In addition, we identified 3 lines that suppress the Nile red but not dauer formation phenotype of *daf-1(m40)* animals (Figure 1). We decided to map these suppressors and named them *ft7*, *ft9*, and *ft10*. We crossed the *ft7*, *ft9*, and *ft10*

suppressors into the *daf-7(e1372)* background to determine that they suppress phenotypes of both TGF- β ligand and receptor.

daf-7(e1372); ft7 and *daf-7(e1372); ft10* animals have Nile red staining that is much brighter than *daf-7(e1372)* or wild type animals (Figure 2). Although *ft7* and *ft10* strongly suppress the Nile red phenotype of *daf-7(e1372)* animals, the effects of these mutations on the Sudan black phenotype of *daf-7(e1372)* animals is less clear. In the several rescreens performed, we identified both high fat and partially suppressed animals in *daf-7(e1372); ft7* and *daf-7(e1372); ft10* populations (Figure 3B and data not shown). We also observed that *daf-7(e1372); ft7* and *daf-7(e1372); ft10* animals are slow-growing and may form more dauers or partial dauers than *daf-7(e1372)* alone. Dauer stage animals store more fat than L4 larval animals of the same genotype. We analyze L4 animals to determine fat level in Sudan black assays; thus, the increased dauer formation phenotype may complicate our analysis of *daf-7(e1372); ft7* and *daf-7(e1372); ft10*.

In order to map the suppressors in the *daf-7(e1372)* background we crossed *daf-7(e1372)* animals four times into the *C. elegans* Hawaiian mapping strain. Using SNP mapping, we determined that the *ft7* and *ft10* mutations are located on the right arm of chromosome II (Jakubowski and Kornfeld 1999; Davis, Hammarlund et al. 2005). *daf-7(e1372); ft7* and *daf-7(e1372); ft10* failed to complement each other, indicating that *ft7* and *ft10* are likely to be alleles of the same gene. We mapped *ft7* to a small genetic region between markers 14.38 and 14.81 on chromosome II containing ~20 genes. One gene in this region is known to result in increased Nile red staining, and was identified in a Nile red suppressor screen of *tph-1* serotonin synthesis defective mutants (K. Jones, personal communication). We sequenced the F29C12.3 gene in *ft7* animals and

identified a G to A mutation that changes a tryptophan to an early stop codon in the protein (Figure 3F). Because this mutation introduces a stop codon in the third exon, we predict that it is a null allele of F29C12.3, also known as *lpo-6* (Figure 3F). We did not determine the molecular identity of the mutation in *ft10*.

Gain of function mutations in G_q signaling suppress fat accumulation in *daf-1(m40)* and *daf-7(e1372)* animals.

The *ft9* suppressor increases the intensity of Nile red staining of *daf-7(e1372)* animals, and suppresses the increased fat storage phenotype of *daf-1(m40)* and *daf-7(e1372)* mutations as assayed by Sudan black (Figure 2, Figure 3A, and Figure 4B). We observed that *daf-7(e1372); ft9* animals lay eggs constitutively, move in a loopy manner (or move with exaggerated bends), and are hyperactive as adults and dauer animals. The *ft9* mutation is also semi-dominant. We initially mapped the *ft9* mutation to the left arm of chromosome I using the Hawaiian *daf-7(e1372)* strain. We later mapped *ft9* using the dominant loopy and hyperactive movement phenotype, rather than suppression of *daf-7(e1372)* Nile red phenotype. We determined that the *ft9* mutation lay between markers at -19 and -12 on chromosome I. The *ft9* mutation causes many phenotypes also produced by alterations in G_o and G_q G-protein signaling (Hajdu-Cronin, Chen et al. 1999; Bastiani, Gharib et al. 2003; Schade, Reynolds et al. 2005). Gain of function mutations in $G\alpha_q$ protein, *egl-30*, result in hyperactivity, loopy movement, and egg-laying constitutive phenotypes (Bastiani, Gharib et al. 2003; Schade, Reynolds et al. 2005), and is located on chromosome I at -12.5. We sequenced *egl-30* in *daf-7(e1372); ft9* animals, and identified a G to T nucleotide base change in the 5th exon of *egl-30a*. This mutation results in a glutamic acid to aspartic acid substitution (Figure 3C-D).

While this mutation results in a substitution of one acidic amino acid for another, the glutamic acid is highly conserved in other G_q proteins (Figure 3E). We also sequenced other components of G-protein signaling on chromosome I including the $G\alpha_o$ *goa-1* subunit and $G\beta$ *gpb-2* subunit, and did not find any mutations in *ft9* animals (Hajdu-Cronin, Chen et al. 1999; Robatzek, Niaccaris et al. 2001).

G-protein coupled receptors (GPCR's) signal through heterotrimeric G-proteins consisting of $G\alpha$, $G\beta$, and $G\gamma$ subunits. *C. elegans* has 17 $G\alpha$ subunits, 2 $G\beta$'s, and one $G\gamma$ subunit (Bargmann 1998). G_q signaling is mediated by the $G\alpha_q$ protein *egl-30*, which activates phospholipase $C\beta$ encoded by *egl-8* (Brundage, Avery et al. 1996; Miller, Emerson et al. 1999). Activation of *egl-30* and *egl-8* results in the conversion of phosphatidylinositol 4, 5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (Berridge 1984). G_o signaling requires the $G\alpha_o$ protein *goa-1*, which activates *dgk-1* diacylglycerol kinase that inhibits accumulation of DAG (Segalat, Elkes et al. 1995; Hajdu-Cronin, Chen et al. 1999).

In this manner, *C. elegans* G_o and G_q pathways act in opposition to each other (Miller, Emerson et al. 1999). Gain of function mutations in G_q components such as *egl-30* phenocopy loss of function mutations in G_o components (Miller, Emerson et al. 1999; Bastiani, Gharib et al. 2003). We identified a gain of function mutation in the G_q protein *egl-30* as a suppressor of *daf-7(e1372)* (Figure 4B-C). Similarly, we find that loss of function mutations in G_o signaling proteins *goa-1* and *dgk-1* also suppress the high fat phenotype of *daf-7(e1372)* (Figure 4B-C).

Neuropeptides, dopamine, and acetylcholine do not regulate fat accumulation of *daf-7(e1372)* animals

Many neurotransmitters and peptides signal through GPCRs to regulate behaviors including pharyngeal pumping, egg-laying, and locomotion. Neurotransmitters such as dopamine, serotonin, acetylcholine, glutamate, and GABA, as well as neuropeptides, signal through GPCR's (Segalat, Elkes et al. 1995; Bargmann 1998; Lackner, Nurrish et al. 1999; Chase, Pepper et al. 2004). In order to determine which GPCR's may mediate the accumulation of fat in *daf-7(e1372)*, we determined the role of neuropeptides and neurotransmitters in regulated fat storage.

C. elegans has ~250 predicted neuropeptides, of which 25% are processed by the proprotein convertase EGL-3 (Husson, Clynen et al. 2006; Husson, Janssen et al. 2007). Deletion mutation in *egl-3* did not affect fat accumulation in *daf-1(m40)* animals (data not shown). Surprisingly, *daf-7(e1372); egl-3(gk238)* animals formed dauers constitutively at all temperatures and could not be analyzed.

Loss of function mutation in *goa-1* and *dgk-1* or gain of function mutation in *egl-30* results in hypersensitivity to the acetylcholinesterase inhibitor aldicarb (Miller, Emerson et al. 1999). Addition of aldicarb increases the amount of acetylcholine in the synapse, and hypersensitivity to aldicarb is thought to indicate excess cholinergic signaling (Miller, Emerson et al. 1999). We added aldicarb to *daf-7(e1372)* to determine if additional cholinergic signaling would suppress the fat storage phenotype of these animals. We did not observe any change in Nile red intensity in *daf-7(e1372)* animals treated with 10µM, 25µM or 100µM aldicarb (data not shown). However, at higher concentrations of aldicarb (100µM) we observed severe growth delay in *daf-7(e1372)* compared to wild type, suggesting that *daf-7(e1372)* may be hypersensitive to aldicarb (data not shown). Furthermore, mutations in the vesicle acetylcholine transporter, *unc-*

17, (Alfonso, Grundahl et al. 1993) did not suppress the Nile red phenotype of *daf-7(e1372)*. Thus, cholinergic signaling does not regulate fat accumulation in the TGF- β signaling pathway.

High levels of exogenous dopamine paralyze *C. elegans*, but loss of function mutations in *goa-1* and *dgk-1* or gain of function mutation in *egl-30* are resistant to addition of exogenous dopamine (Chase, Pepper et al. 2004). Animals defective in dopamine synthesis due to mutation in the tyrosine hydroxylase *cat-2* are also resistant to the effects of exogenous dopamine (Lints and Emmons 1999; Chase, Pepper et al. 2004). However, loss of dopamine by the *cat-2(e1112)* mutations does not suppress the fat accumulation of *daf-7(e1372)* animals (data not shown).

Metabotropic glutamate receptors regulate fat accumulation in *daf-7(e1372)* animals

In contrast, we find that deletion mutations in the vesicular glutamate transporter *eat-4* (Lee, Sawin et al. 1999) partially suppress the fat accumulation phenotype of *daf-7(e1372)* animals (Figure 4B-C). Mutations in *eat-4* do not suppress the dauer formation phenotype of *daf-7(e1372)*. In addition, *eat-4(ky5)* animals have reduced pharyngeal pumping. Interestingly, *eat-4* mutations suppress the bordering phenotype of *daf-7(e1372)*.

C. elegans has three metabotropic glutamate receptors: *mgl-1*, *mgl-2*, and *mgl-3* (Dillon, Hopper et al. 2006). Thus far, mutation of these receptors has not been associated with any *C. elegans* phenotypes. We determine that mutations in both *mgl-1* and *mgl-3* result in a partial suppression of *daf-7(e1372)* fat accumulation, yet do not alter fat storage on their own (Figure 4B-C). *daf-7(e1372); mgl-1(tm1811); mgl-3(tm1766)* triple mutants have a further reduction in fat stores compared to *daf-7(e1372)*;

mgl-1(tm1811) or *daf-1(e1372); mgl-3(tm1766)*, suggesting that these metabotropic glutamate receptors act in parallel to regulate fat accumulation (Figure 4B-C). *mgl-1* and *mgl-3* mutations do not alter the bordering, pharyngeal pumping, egg-laying, or dauer formation phenotypes of *daf-7(e1372)* animals (Figure 4A and data not shown). Loss of TGF- β signaling results in excess glutamate signaling through metabotropic glutamate receptors that results in fat accumulation.

We fused the *mgl-1* promoter to mCherry and the *mgl-3* promoter to GFP to determine which cells express these receptors. *pmgl-1::mCherry* expresses in AIA amphid interneurons, RMDV and RMDD ring interneurons/motor neurons, and the serotonergic pharyngeal neuron NSM (Figure 4D). *pmgl-3::GFP* promoter reporter fusion is also expressed in NSM as well as ADF, ASE, and AWC amphid sensory neurons, and the RIB and RIC interneurons (Figure 4D). We observe variable expression of *mgl-3::GFP* in BAG ciliated neurons. The largely non-overlapping expression patterns of these reporter fusions were consistent with redundant functions of *mgl-1* and *mgl-3* in mediating excess fat of *daf-7(e1372)* mutants.

Excess fat content of *daf-7* mutant animals is associated with increased fat synthesis

Our results indicated that excess fat accumulation in *daf-7* and *daf-1* mutants was independent of processes that regulate feeding, egg-laying, and growth. We therefore sought to determine the consequences of *daf-7/daf-1* pathway inactivation on metabolism. We focused on fat synthesis in wild type and mutant strains. By following the incorporation of ¹³C labeled carbons into palmitate, stearate, and vaccenic acids extracted from *C. elegans* lysates, we obtained estimates for the fraction of total lipids that were synthesized *de novo*. Relative to wild type, fat synthesis was upregulated

nearly ~3 fold in *daf-1(m40)* mutants (Figure 5A). This upregulation was abolished in *daf-1(m40); daf-3(mgDf90)* mutants (Figure 5A).

To further investigate the role of *de novo* fat synthesis in excess fat of *daf-7(e1370)* and *daf-1(m40)* mutants, we used RNAi to inactivate F32H2.5, encoding fatty acid synthase (*fasn-1*). Fatty acid synthase carries out the cyclical reactions that generate longer chain fats from malonyl-CoA (Asturias, Chadick et al. 2005). Exposure of wild type, *daf-7(e1372)* and *daf-1(m40)* animals to *fasn-1* RNAi resulted in larval growth arrest (example in Figure 5B). Growth arrested *daf-7(e1372)* and *daf-1(m40)* animals had reduced fat levels indistinguishable from growth arrested wild type animals (Figure 5B-C and data not shown). Animals that did not arrest accumulated fat, suggesting the developmental delay is due to an inability to store fat. Finally, we determined that a ~6kb predicted *fasn-1* promoter-reporter fusion was expressed in hypodermal skin cells (Figure 5D). This expression pattern was consistent with the metabolic role of *fasn-1* since hypodermal and intestinal cells are major sites of fat storage and metabolism in *C. elegans* (Kimura, Tissenbaum et al. 1997; McKay, McKay et al. 2003; Mak, Nelson et al. 2006).

Discussion

We determined that loss of function mutations in G_o signaling and gain of function mutation in G_q signaling suppress fat accumulation in *daf-7(e1372)*, without altering dauer formation (Figure 6). In part, this is due to signaling through metabotropic glutamate receptors expressed in head neurons. Mutations in G_o and G_q pathway components suppress fat accumulation in *daf-7(e1372)* to a greater extent than loss of glutamate signaling. We are unable to determine if this is due to the involvement of other

GPCR's or other phenotypes of *egl-30* gain of function mutations such as hyperactivity. These signals eventually regulate fatty acid synthesis in the hypodermis and possibly intestine of *C. elegans* (Figure 6).

Although we sought to identify signals from the interneurons RIM and RIC regulating fat storage, it is unlikely that RIM and RIC contain glutamate. The vesicular glutamate transporter *eat-4* required for glutamate signaling is expressed in many head neurons but not RIM or RIC (Lee, Sawin et al. 1999). This suggests that RIM and RIC signal to glutamate cells using an as of yet unidentified neurotransmitter or peptide. It is also possible that glutamate signaling acts in parallel to TGF- β signaling to regulate fat storage.

The neurons expressing metabotropic glutamate receptors eventually must communicate with the site of fat storage and synthesis in the periphery of the animal. This could occur by signaling through another neurotransmitter or peptide. However, another possible mechanism is through lipid signals produced by metabotropic glutamate signaling. Retrograde signaling in the hippocampus and other brain regions is mediated by metabotropic glutamate receptors on the postsynaptic cell (Piomelli, Astarita et al. 2007). Signaling through mGluR's leads to the accumulation of DAG, which can be converted to 2-arachidonoyl-*snglycerol* (2-AG) by DAG lipases. 2-AG is an endogenous endocannabinoid that can cross the synaptic cleft and activate cannabinoid receptors on the presynaptic cell, modulating release of glutamate (Piomelli, Astarita et al. 2007). 2-AG can also be converted to prostanoids, which can act in a paracrine manner to regulate receptors (Piomelli, Astarita et al. 2007). If this occurs in *C. elegans*, we would predict

that increased signaling through 2-AG or prostanioids derived from 2-AG will suppress the fat accumulation in TGF- β mutants.

GPCR signaling and metabotropic glutamate signaling in obesity

In mammals, several key GPCR's regulate energy homeostasis downstream of leptin and insulin signaling. Leptin signaling in POMC and NPY/AgRP neurons increases signaling through the GPCR, melanocortin 4 receptor (MC4R), leading to changes in energy expenditure and food intake (Flier 2004). Mutations in MC4R result in profound obesity (Flier 2004). Melanin concentrating hormone (MCH) neurons in the lateral hypothalamus receive projections from POMC and NPY/AgRP neurons. MCH also binds to a GPCR, MCHR, and loss of either results in a lean hypophagic mouse (Flier 2004). In addition, deletion of the metabotropic glutamate receptor mGlu5 results in weight loss, which is not accompanied by changes in food intake (Bradbury, Campbell et al. 2005). As in mammals, signaling through GPCR's functions downstream of *C. elegans* peptides that monitor energy availability.

Role of hypothalamic glutamate

Much of synaptic activity in the brain can be attributed to the actions of the predominantly excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA. The hypothalamus is no exception. Blocking glutamate and GABA signals eliminates most if not all miniature, and evoked activity in hypothalamic slices (van den Pol 2003). NPY/AgRP neurons promote food intake and are thought to contain GABA, while POMC neurons decrease food intake and may contain glutamate (Collin, Backberg et al. 2003; van den Pol 2003).

Recent evidence suggests that leptin plays a role in regulating plasticity of GABAergic and glutamatergic transmission in hypothalamic neurons. Animals lacking leptin have more excitatory synapses on NPY/AgRP neurons and fewer excitatory synapses on POMC neurons. This effect can be reversed by addition of leptin (Horvath and Diano 2004; Pinto, Roseberry et al. 2004). Modulation of the number of excitatory glutamate and inhibitory GABA synapses on POMC and NPY/AgRP neurons may alter the excitatory or inhibitory tone of these neurons throughout the day, thus regulating food intake and energy homeostasis (Horvath and Diano 2004). *C. elegans* are thought not to have synaptic plasticity, but we find that glutamate signaling through metabotropic glutamate receptors promotes fat storage in the TGF- β pathway.

Figure 1: EMS mutagenesis screen and rescreens

~1000 late L4 *daf-1(m40)* animals were treated with EMS to introduce mutations.

We screened 125,000 F₂ progeny for suppression of the *daf-1(m40)* Nile red phenotype.

We rescreened the Nile red phenotype of mixed stage F₃ animals. We split F₄ animals into several groups in order to analyze dauer formation at 26.5 °C and stain animals with Sudan black and Nile red.

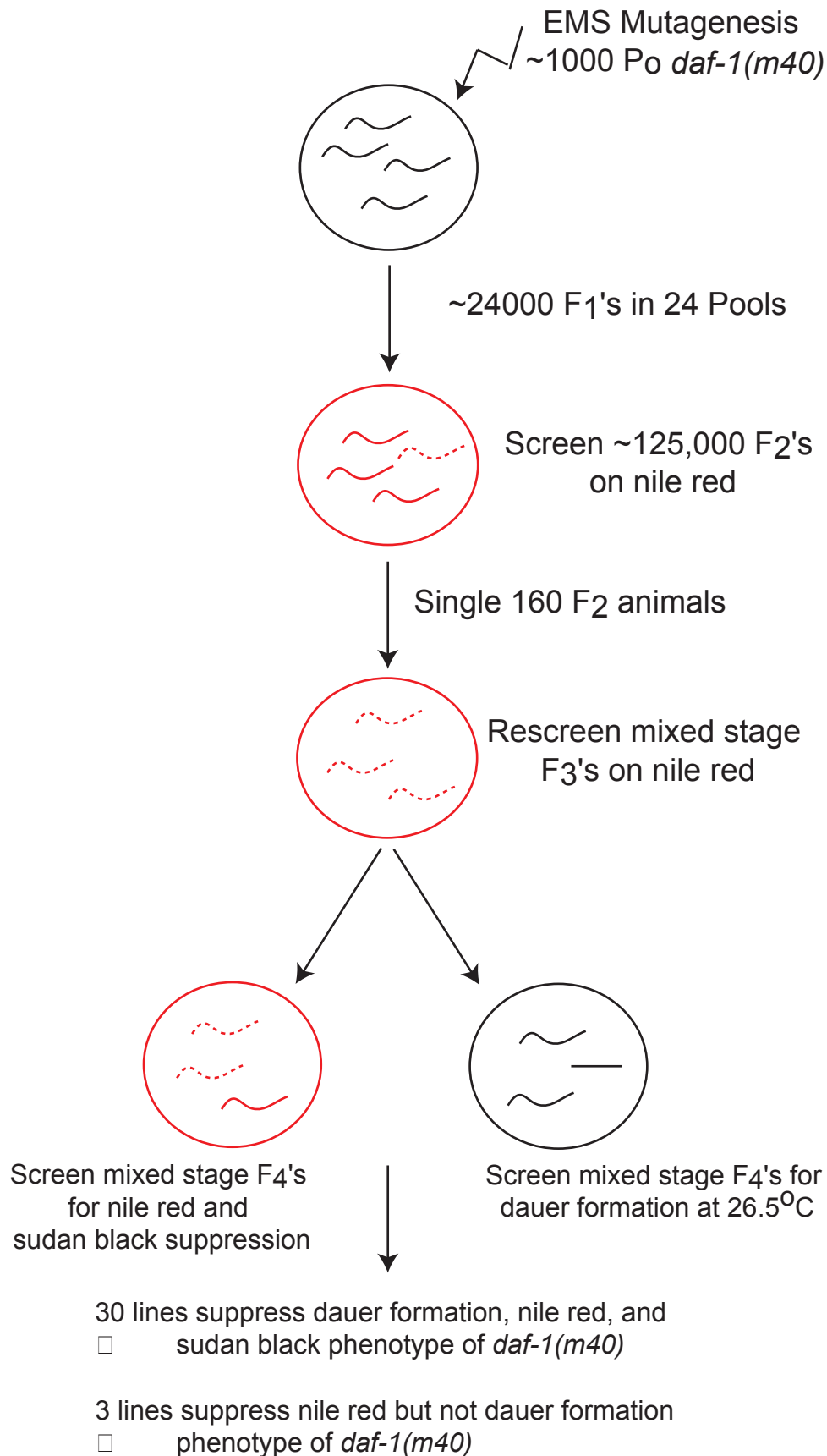


Figure 2: 3 mutations suppress the Nile red but not dauer formation phenotype of *daf-7(e1372)* animals

Nile red staining of L4 larval stage animals. *daf-7(e1372)* animals have decreased Nile red staining compared to wild type. This phenotype is suppressed by *ft7*, *ft10*, and *ft9* mutations. *daf-7(e1372); ft7* and *daf-7(e1372); ft10* mutants have more Nile red staining than wild type or *daf-7(e1372)*. Nile red intensity in *daf-7(e1372); ft9* animals is similar to wild type.

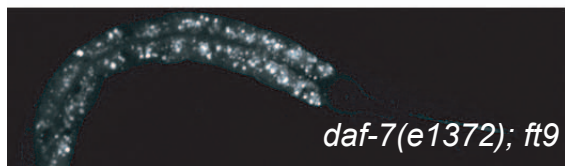
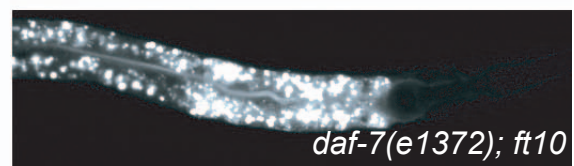
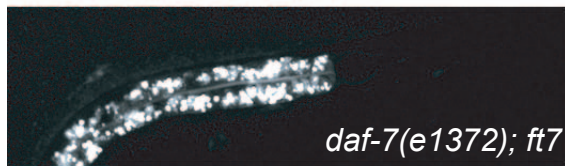
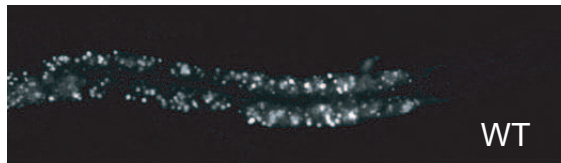
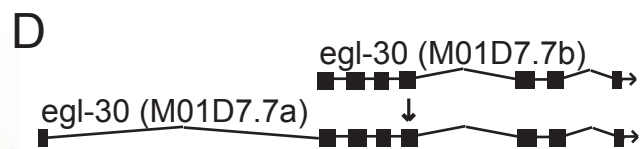
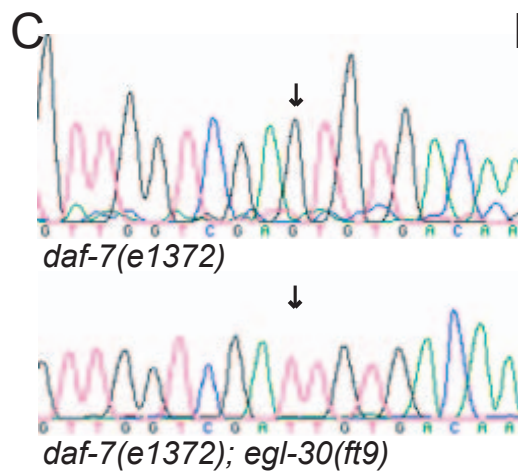
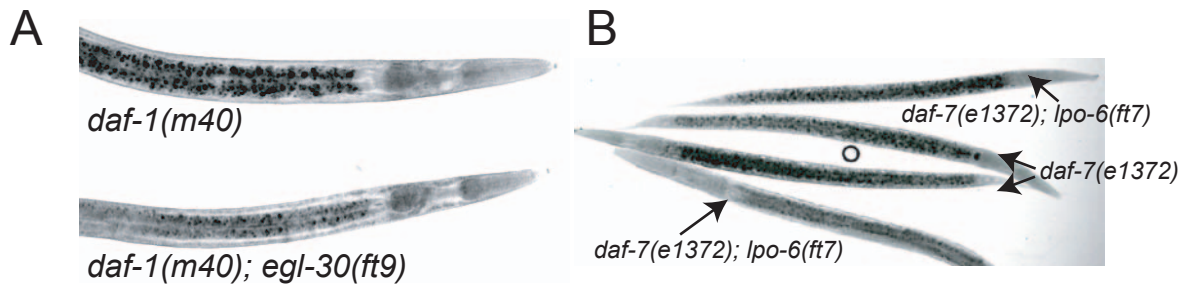


Figure 3: Cloning of *daf-1(m40)* and *daf-7(e1372)* suppressors

- (A) *ft9* mutation suppresses the sudan black phenotype of *daf-1(m40)* animals
- (B) Co-stained *daf-7(e1372)* and *daf-7(e1372); ft7* animals. *daf-7(e1372)* have dark sudan black staining and mutation in *ft7* sometimes appears to suppress this phenotype (animals in lower left corner) while other *ft7* animals resemble *daf-7(e1372)* animals (upper right corner).
- (C-D) *ft9* mutation results in G to T amino acid change in the 5th exon of *egl-30* (M01D7.7a) which results a glutamic acid to aspartic acid amino acid substitution.
- (E) Alignment of *C. elegans egl-30* to other G_q proteins. Glutamic acid mutated in *ft9* is highly conserved in other species.
- (F) *ft7* mutation results in a nucleotide change of G to A accompanies by introduction of an early stop codon in exon 3 of *lpo-6* (F29C12.3).



E

<i>C. remanei</i>	224	FLValseyDQ	VLVECDNENR	MEESKALFRT	IITYPWFTNS
<i>C. briggsae</i>	224	FLValseyDQ	VLVECDNENR	MEESKALFRT	IITYPWFTNS
<i>B. mori</i>	222	FLValseyDQ	ILFESDNENR	MEESKALFKT	IITYPWFQHS
<i>D. melanogaster</i>	222	FLValseyDQ	ILFESDNENR	MEESKALFRT	IITYPWFQNS
<i>M. gallopavo</i>	228	FLValseyDQ	VLVESDNENR	MEESKALFRT	IITYPWFQNS
<i>H. sapiens</i>	228	FLValseyDQ	VLVESDNENR	MEESKALFRT	IITYPWFQNS
<i>C. elegans</i>	224	FCVAMSEYDQ	VLHEDETNR	MHESLKLFD	ICNNKWFTDT
<i>S. cerevisiae</i>	319	FCVSLSEYDQ	TLMEQKNQNR	FQESLVLFDN	IVNSRWEART

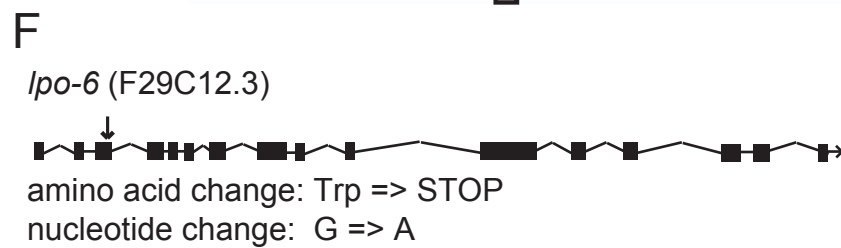


Figure 4: GPCR and metabotropic glutamate signaling are required for fat increase but not feeding reduction of *daf-7(e1372)* mutants

(A) Mutations in *mgl-1* or *mgl-3* do not alter the pharyngeal pumping rate of *daf-7(e1372)* (asterisk indicate $p < 0.001$ determined by ANOVA with Bonferroni post-test compared to *daf-7* mutants). Mutations in *dgk-1*, *goa-1*, or *eat-4* reduce pharyngeal pumping rate in well-fed conditions.

(B-C) fat content of *daf-7(e1372)* animals. Increased fat but not reduced feeding rate of *daf-7(e1372)* was suppressed by mutations in *goa-1*, *dgk-1*, *eat-4*, *mgl-1*, and *mgl-3* as well as a predicted gain of function mutation in *egl-30*. Asterisks indicate statistical significance relative to *daf-7* as determined by t-test. Standard deviation bars are shown.

(D) Transcriptional reporters fused to putative promoters of *mgl-1* and *mgl-3* were each expressed in only limited number of neurons identified by arrowheads. Both *mgl-1* and *mgl-3* are expressed in NSM pharyngeal neurons.

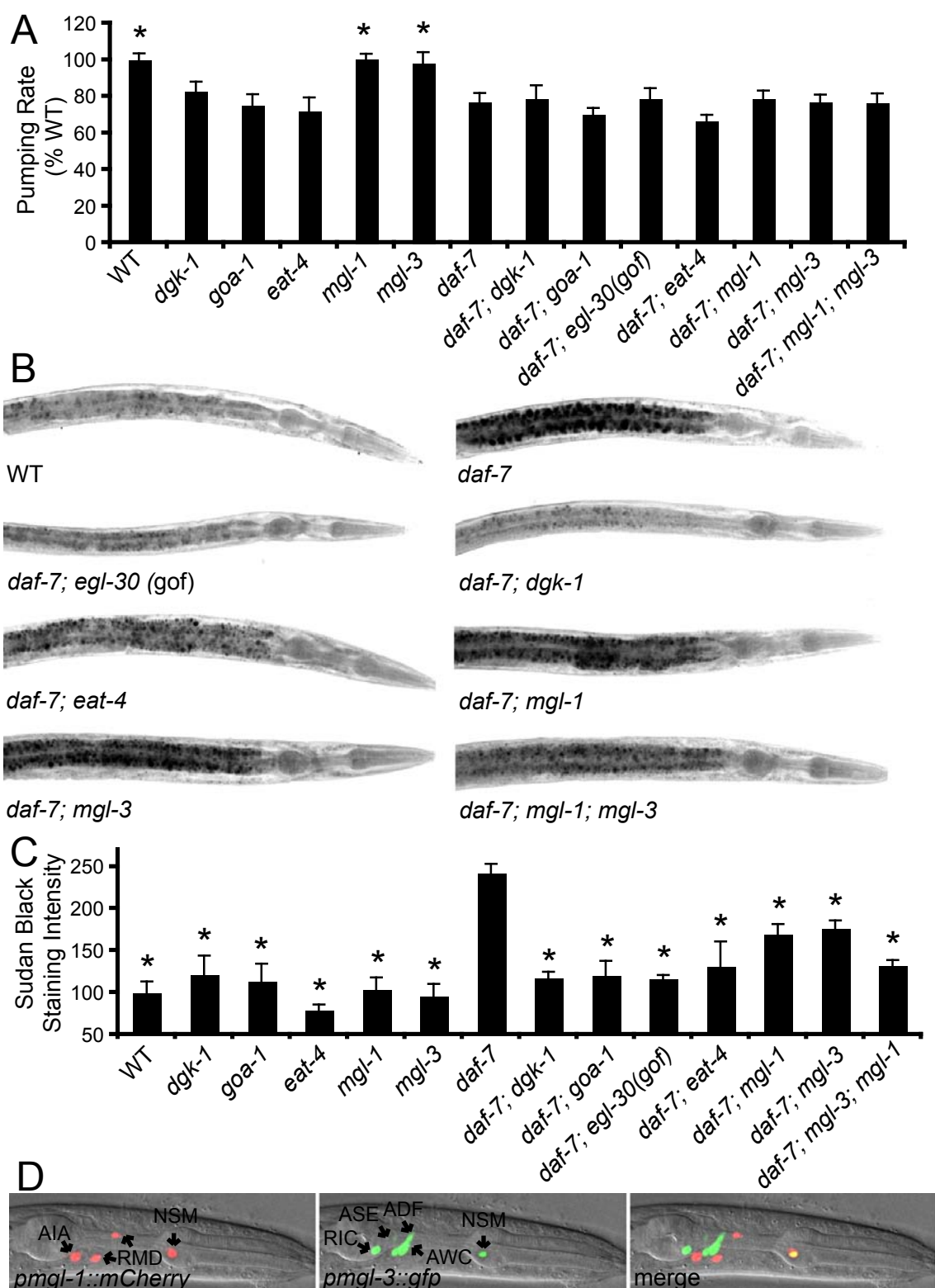


Figure 5: Fat synthesis is upregulated in *daf-1(m40)* animals.

(A) *de novo* fat synthesis was upregulated in *daf-1(m40)* and was dependent on *daf-3*.

Standard error bars are shown.

(B-C) RNAi of *fans-1* resulted in developmental arrest and fat reduction in both wild type and *daf-1(m40)* animals. Standard error bars are shown.

(D) A 6kb *promoter::gfp* for *fasn-1* showed expression in the hypodermis, a key peripheral fat metabolic tissue in *C. elegans*.

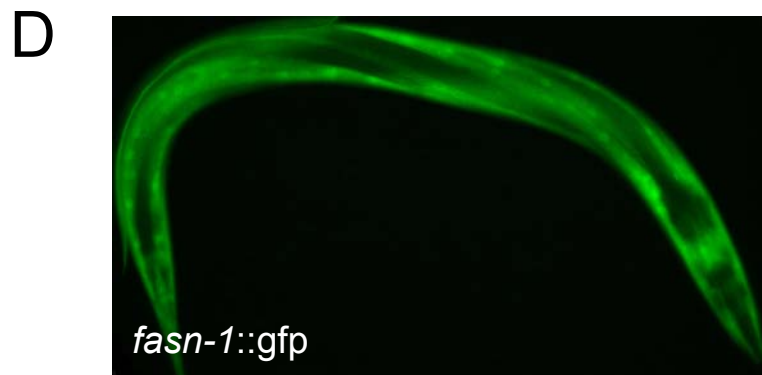
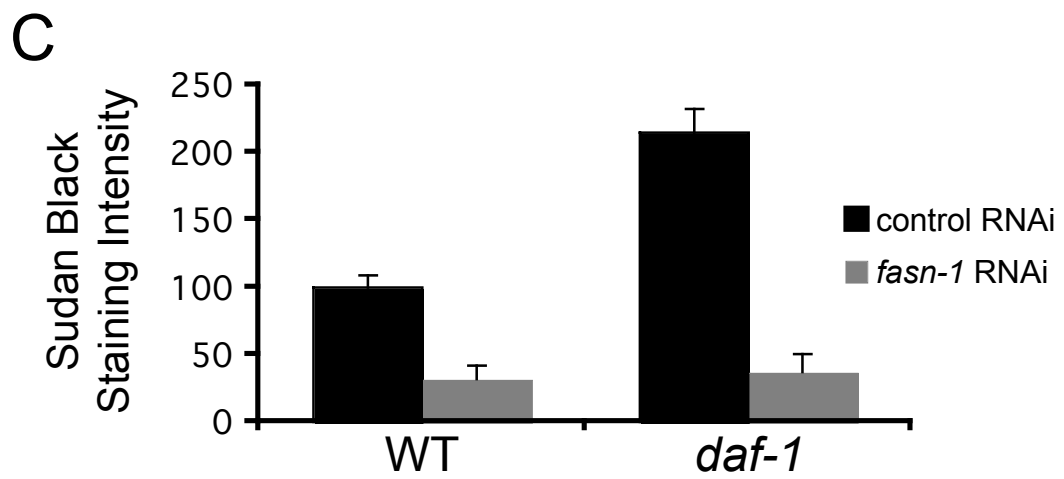
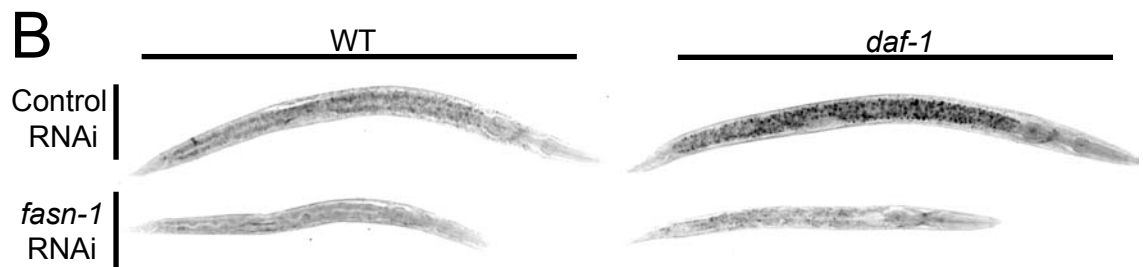
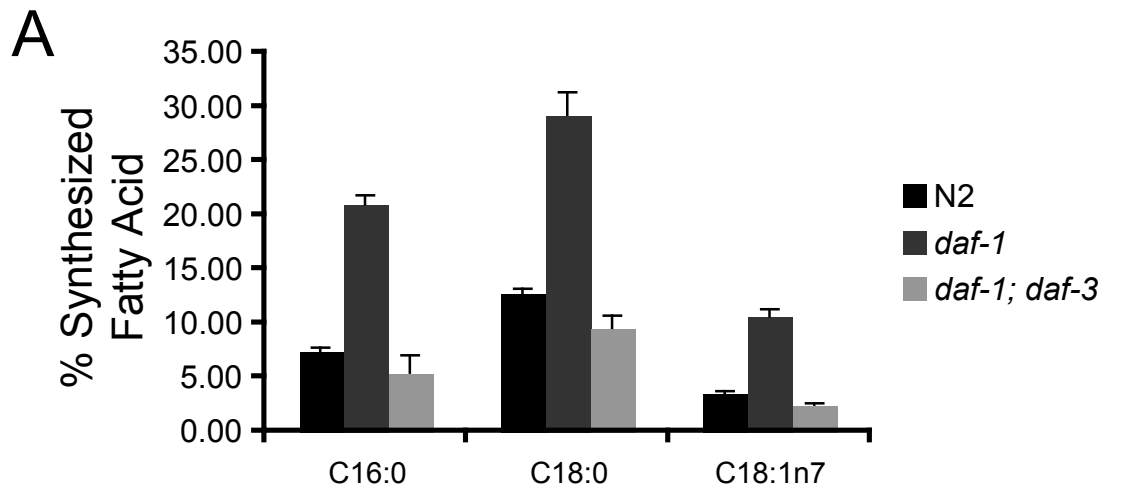
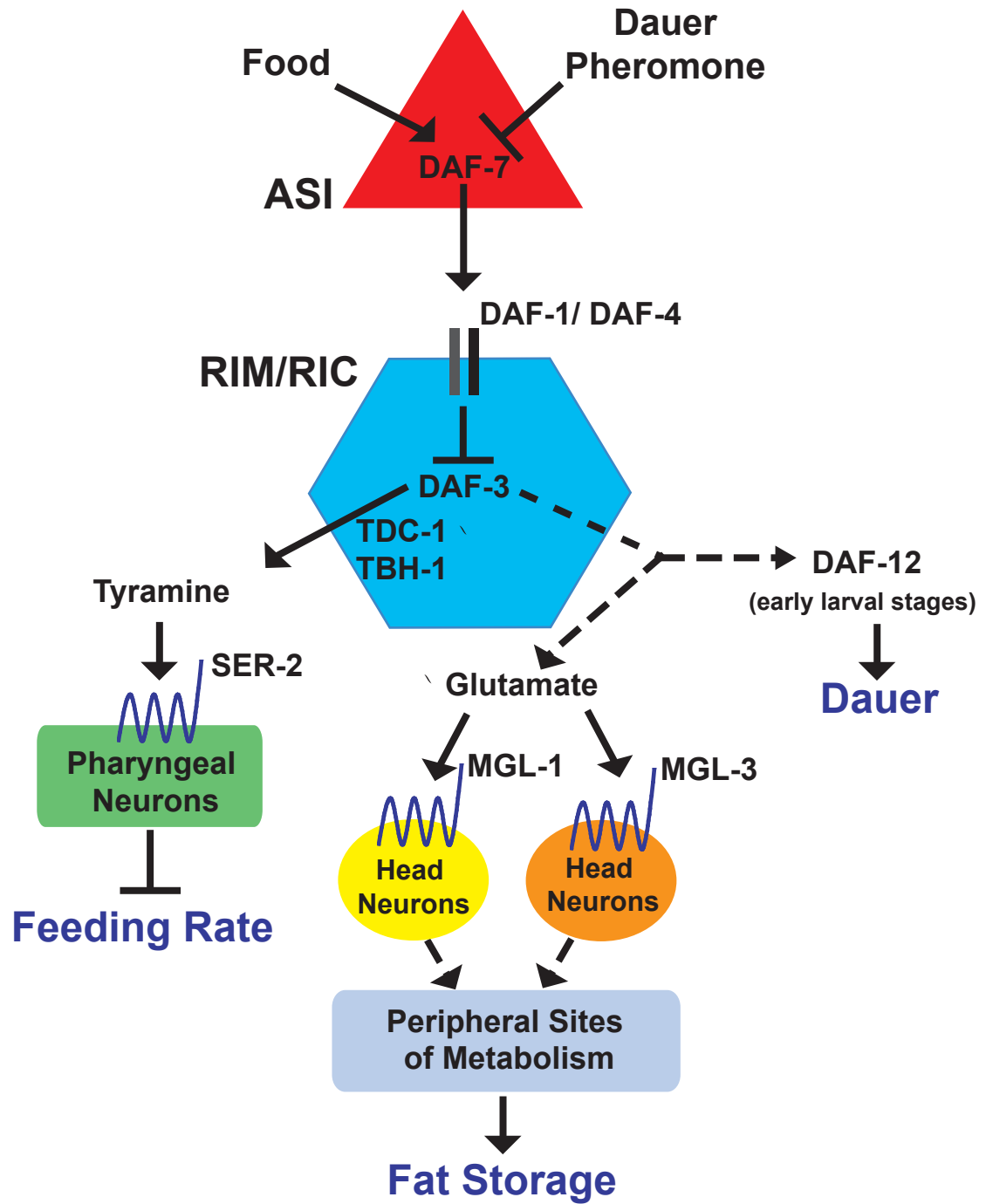


Figure 6: Model for TGF- β regulation of fat storage

Glutamate signaling through neuronally expressed metabotropic glutamate receptors, MGL-1 and MGL-3, partially mediates fat increasing effects of *daf-7* inhibition. Fat increase in *daf-7(-)* animals is ultimately associated with increased *de novo* fat synthesis in peripheral sites of fat storage and utilization. Dotted lines indicate unknown signal emanating from RIM and RIC.



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Chapter VI: Initial characterization of feeding regulatory genes in *C. elegans*

Introduction

All animals must take in nutrients from the environment. Feeding behavior is not only required for life but can be extremely rewarding. Studies in humans and rodents suggest that feeding impinges upon the same reward pathways in the brain activated by addictive drugs (Volkow and Wise 2005). Feeding behavior is regulated by internal signals of nutritional requirements and external sensation of food (Volkow and Wise 2005; Morton, Cummings et al. 2006). As in mammals, the presence of food in the environment and the experience of starvation alter many *C. elegans* behaviors. In the absence of food, animals will retain eggs, reduce feeding rate, and initiate foraging behavior (Trent 1982; Avery and Horvitz 1990; Hills, Brockie et al. 2004). Food is a strong motivator in *C. elegans* and can be used to entrain animals in associative learning paradigms (Sawin, Ranganathan et al. 2000; Nuttley, Atkinson-Leadbetter et al. 2002; Chao, Komatsu et al. 2004).

Feeding and metabolism are regulated by the availability of food (Avery and Horvitz 1990; Van Gilst, Hadjivassiliou et al. 2005). *C. elegans* feed using a neuromuscular pump called the pharynx. The pharynx ingests, concentrates, and grinds up bacteria before propelling it into the gut (Avery 1993; Avery and Shtonda 2003). Although this behavior is constitutive, it is strongly modulated by the presence of food. In the absence of food, *C. elegans* feed at a slower rate. When reintroduced to food, animals that have experienced starvation will increase their feeding rate beyond that of well-fed animals (Avery and Horvitz 1990). Furthermore, food-deprived animals will feed at lower concentrations of food than well-fed animals (Avery and Horvitz 1990). In

C. elegans, pathways important in food sensation such as serotonin signaling also regulate feeding (Sze, Victor et al. 2000).

Although pharyngeal pumping is modulated by the presence of food and the experience of starvation, screens to identify genes regulating pharyngeal pumping have focused on identifying animals with mechanical defects in the pharynx (Avery 1993). Mutations in such “eat” genes were isolated based on producing a starved appearance or developmental delay. Several “eat” genes encode for proteins involved in the mechanical workings of the pharyngeal neuromuscular pump such as glutamate and acetylcholine signaling. In contrast, animals defective in serotonin signaling which indicates the presence of food have less severe defects in pharyngeal pumping and lack the starved appearance of “eat” mutants. Uncovering genes that modulate pharyngeal pumping without affecting the core pharyngeal machinery will likely shed light on additional pathways involved in regulating pharyngeal pumping in dynamic environments.

A genome-wide RNAi screen uncovered over 400 genes that regulate the accumulation of a lipophilic vital dye, Nile red, thought to be indicative of fat content in *C. elegans* (Ashrafi, Chang et al. 2003). These genes encode enzymes important in metabolism, transcription factors, and signaling molecules. In order to discover genes involved in pathways that coordinate perception of food with the fat metabolism of the animal, we used RNAi to screen the 400 genes identified to regulate fat storage for differences in feeding rates. Interestingly, only ~30 of the 400 genes that regulate fat content also regulate the feeding rate of well-fed animals. These genes highlight the importance of *C. elegans* nervous system especially the sensory neuron ASI in regulating feeding behavior and fat storage.

Results

Identification of feeding regulatory genes in *C. elegans*

To determine whether *C. elegans* fat regulatory genes impact food intake behavior, we measured pharyngeal pumping rates of animals exposed to RNAi of each of 400 genes that impact fat accumulation in the intestinal cells of animals (Ashrafi, Chang et al. 2003). We assayed pharyngeal pumping rates of well-fed young adult animals grown on each of the control or test RNAi clones starting with synchronized first larval stage. Using this strategy, we found that only 28 of the 400 fat regulatory genes also impact pumping rate (Figure 1A). RNAi inactivation of 10 of these genes produced an increase in pharyngeal pumping rate accompanied by a decrease in Nile red staining intensity (Figure 1A and (Ashrafi, Chang et al. 2003). RNAi inactivation of 9 genes resulted in both decreased pharyngeal pumping and Nile red staining (Figure 1A-B). Of these genes five are predicted to act as structural or developmental components of the pharynx and were not studied further (Figure 1B). RNAi inactivation of 9 genes produced increased Nile red staining of these, one gene resulted in increased pharyngeal pumping rate while the others resulted in decreased pharyngeal pumping rate (Figure 1A). The magnitude of change in pharyngeal pumping rate is comparable to the reduction in pumping rate noted in mutant animals that cannot synthesize serotonin, a neurotransmitter postulated to mediate food sensation in *C. elegans* (Sze, Victor et al. 2000).

These feeding regulatory genes are predicted to encode proteins that have a wide range of biological functions including kinases, GPCR's, sugar binding proteins, metabolic genes, and potassium channels (Table 1).

Expression pattern of feeding regulatory genes

To determine potential *in vivo* sites of action of these feeding regulatory genes we constructed transcriptional and translational GFP fusion reporters for most of these genes that utilized their endogenous upstream genomic sequences. *tag-160*, *prk-2*, *cnt-1*, *tax-6*, and F13B6.2 increase pharyngeal pumping rate when RNAi'd and are expressed in the pharyngeal muscle (Figure 2A) and (Kuhara, Inada et al. 2002). *cnt-1* and *tax-6* also have broad neuronal expression in addition to expression in the pharyngeal muscle (Figure 2A).

Many feeding regulatory genes are expressed in head and pharyngeal neurons. ZK39.7, T05C12.1, *srw-100*, and F56H11.6 are expressed exclusively in the ciliated sensory neuron ASI (Figure 2B). *nkat-1*, Y49A3A.1, K02E7.11, and Y11D7A.8 are also expressed in ASI in addition to a few additional neurons. Y11D7A.8 and Y49A3A.1 are expressed in ASI and unidentified labial neurons. K02E7.11 is expressed in ASI as well as pharyngeal neurons NSM, I1, and M1. Translational GFP fusions of full-length *nkat-1* showed variable expression sometimes restricted to ASI and sometimes including additional unidentified neurons (Figure 2B). T21C9.11 and *gpd-3* are broadly expressed in the nervous system (Figure 2B and data not shown). *glr-7* and C09G12.5 are expressed in the serotonergic pharyngeal neuron NSM (Figure 2B and data not shown) however *glr-7* has additional expression in I1, 12, 16, and M1 pharyngeal neurons (Brockie, Madsen et al. 2001). F26H9.4 is expressed in the sensory neuron ADL and unidentified labial neurons (Figure 2B). *hlh-15* is expressed in the interneuron AVK in addition to several neurons in the tail (A. Kahn-Kirby personal communication).

Expression analysis reveals the importance of the nervous system, in particular the sensory neurons ASI in regulating pharyngeal pumping and fat storage.

In addition to the pharynx and neurons, feeding regulatory genes were also expressed in the hypodermis. B0310.1 and *dur-1* are both expressed in the hypodermis (or possibly muscle) of the animal (Figure 2C). GFP reporters for *srd-45* and F31F6.2 did not give a consistent expression pattern.

Feeding regulatory genes may interact with dauer pheromone and function in the TGF- β pathway

Several of the feeding regulatory genes are expressed in the sensory neuron ASI. This neuron releases the neuroendocrine TGF- β ligand *daf-7* which is transcriptionally downregulated by high levels of dauer pheromone to initiate a signaling cascade leading to dauer formation (Figure 3A) (Ren, Lim et al. 1996). Dauer pheromone is constitutively secreted by *C. elegans* as a measure of population density (Riddle, Blumenthal et al. 1997). Previous studies have shown that in addition to regulating *daf-7::gfp* dauer pheromone modulates the expression of several chemosensory receptors (Peckol, Troemel et al. 2001). GFP reporter lines for select feeding regulatory genes were exposed as L1's to a crude extract of dauer pheromone and expression levels of the GFP reporters were assayed in L2 or young L3 animals. Preliminary results suggest that addition of dauer pheromone downregulates expression of GFP reporters for ZK39.7, T05C12.1, and K02E7.11 (Figure 3A and data not shown). In contrast, dauer pheromone may increase expression of the GFP reporter for *srw-100* (Figure 3A). Dauer pheromone did not affect the expression levels of GFP reporters for F26H9.4, Y11D7A.8, *nkat-1*, and T21C9.11 (data not shown).

In addition preliminary evidence suggests that several feeding regulatory genes may alter the response of *daf-7::gfp* to pheromone. Exposure of *daf-7::gfp* animals to dauer pheromone results in a decrease in GFP reporter expression. In order to determine if feeding regulatory genes mediate this response we exposed *daf-7::gfp* animals to dauer pheromone and RNAi for each of several feeding regulatory genes. In the absence of pheromone, RNAi of feeding regulatory genes did not affect *daf-7::gfp* expression (data not shown). Exposure of *daf-7::gfp* animals to dauer pheromone and RNAi of ZK39.7, T05C12.1, K02E7.11, and F56H11.6 resulted in a further decrease in *daf-7::gfp* expression (Figure 3B and data not shown). RNAi of B0310.1 or *dur-1* reduces the effect of pheromone on *daf-7::gfp* (Figure 3C). In the presence of pheromone, genes expressed in the hypodermis inhibit *daf-7::gfp* while several genes in ASI promote or protect *daf-7::gfp*. RNAi of *srw-100*, Y11D7A.8, *nkat-1*, and F13B6.1 did not modify the response of *daf-7::gfp* to dauer pheromone. It is unclear if the genes mentioned above interact with *daf-7* in the absence of pheromone or if the epistatic relationships observed only occur in certain environmental conditions.

Feeding regulatory genes interact with *daf-7*/TGF- β , glutamate, and serotonin signaling

The experiments described above suggest that feeding regulatory genes may interact with the *daf-7*/TGF- β pathway. As reported in previous chapters, loss of *daf-1* or *daf-7* results in a decrease in pharyngeal pumping along with increased fat storage. This enabled us to determine if feeding regulatory genes that increase pharyngeal pumping function in the *daf-7*/TGF- β pathway. Mutations in *daf-7* or *daf-1* decreased the pumping rate of all the feeding regulatory genes (Figure 4A). *daf-7* or *daf-1* are epistatic to RNAi

of T05C12.1 suggesting the kinase T05C12.1 acts upstream of *daf-7* in ASI (Figure 4A). Other feeding regulatory genes that increase pharyngeal pumping rate are likely to act in parallel to the *daf-7*/TGF- β pathway.

Mutations in the vesicular glutamate transporter *eat-4* are required for glutamatergic signaling in *C. elegans* and result in a decrease in pharyngeal pumping (Lee, Sawin et al. 1999). To determine if feeding regulatory genes interact with glutamate signaling 10 RNAi's that increase pharyngeal pumping rate were assayed for modulation of *eat-4(ad819)* pumping rate. Mutations in *eat-4* decreased the feeding rate of all the feeding regulatory genes assayed (Figure 4B). Mutations in *eat-4* are epistatic to RNAi of F13B6.2, ZK39.7, and *srd-45* (Figure 4B). The other increased feeding regulatory genes are likely to act in parallel to glutamate signaling to regulate pharyngeal pumping.

Animals lacking serotonin signaling have decreased feeding rates in addition to increase fat stores (Sze, Victor et al. 2000). Likewise, exogenous addition of serotonin increases pharyngeal pumping rate while decreasing fat stores (personal communication S. Srinivasan). RNAi of T21C9.11, *glr-7*, and C09G12.5 did not decrease the pharyngeal pumping rate of serotonin treated animals suggesting that these genes function upstream of serotonin to regulate pharyngeal pumping (Figure 4C). In fact both *glr-7* and C09G12.1 are expressed in the serotonergic NSM pharyngeal neuron suggesting they could modulate serotonin release from this neurons. RNAi of the neuronal kinases Y11D7A.8 and F56H11.6 suppresses the increased pumping effects of serotonin addition (Figure 4C).

Based on these epistasis experiments T05C12.1 interacts with the *daf-7*/TGF- β pathway, F13B6.2, ZK39.7, and *srd-45* regulate pharyngeal pumping through glutamate signaling, and T21C9.11, Y11D7A.8, F56H11.6, *glr-7*, and C09G12.5 function in the serotonergic pathway.

Starvation modulated pharyngeal pumping

The absence of food and experience of starvation modulates pharyngeal pumping. In the absence of food animals reduce their pumping rate while the experience of starvation can elicit pumping behaviors at lower concentrations of bacteria than normal (Avery and Horvitz 1990). Animals that have been starved for two hours then reexposed to food have an increased pumping rate (Figure 5). After five hours of starvation animals no longer have a pumping rate above the basal (well-fed) level. If these animals are allowed to recover on food for one or three hours the pumping rate drops below the basal level (Figure 5). The meaning of these changes is still unclear. One hypothesis is that animals exposed to short periods of starvation may increase their pumping rate when returned to food to increase caloric intake. However after longer periods of starvation animals may become decrepit or undertake adaptations to long periods of starvation and thus respond differently.

In order to determine if feeding regulatory genes mediate this response to starvation, RNAi animals were starved for two or five hours then reexposed to food. After experiencing two hours of starvation control animals increase their pumping rate by 20% when returned to food. In contrast, RNAi's that increase pumping rate do not have a further increase in pumping rate upon reexposure to food after starvation (Figure 5A). However, like untreated animals RNAi's that result in increased pumping rate dip below

basal pumping rate when starved for five hours and let recover on food for one or three hours (Figure 5A).

RNAi's that decrease pharyngeal pumping also respond to the experience of starvation by increasing their pumping rate although T21C9.11 and Y49A3A.1 are still reduced compared to wild type (Figure 5B). Unlike wild type, after five hours of starvation upon reexposure to food many RNAi's with a decrease in feeding rate do not return to their basal pumping rate (Figure 5B).

Mutant phenotypes for genes identified by RNAi to regulate pharyngeal pumping

I obtained mutants for 9 of the 23 feeding regulatory genes. Of these mutants *srd-45* and *cnt-1* failed to produce a pharyngeal pumping phenotype; although *srd-45* did have increased Nile red staining despite the RNAi phenotype of decreased Nile red (Figure 6B and 6D). Furthermore mutations in *srd-45* and *cnt-1* did not suppress the pharyngeal pumping phenotypes of *tph-1* or *daf-7* mutant animals (Figure 6B-C). Mutants for *prk-2*, *srw-100*, and *dur-1* phenocopied the pharyngeal pumping phenotype produced with RNAi of each gene (Figure 6A and 6C). However mutants in *prk-2* did not have altered Nile red staining (Figure 6D). Mutations in *prk-2* partially suppress the pharyngeal pumping phenotype of *daf-1* mutants (Figure 6C). Mutants in *tax-6*, *nkat-1*, *tag-160*, and BO310.1 have the opposite pharyngeal pumping phenotype as that identified by RNAi (Figure 6A and Table 2).

Some chemosensory and neuropeptide genes may affect pharyngeal pumping

Many chemosensory genes do not affect pharyngeal pumping (Figure 7A). Mutations in *osm-12*, *osm-8*, *osm-7*, *che-13*, and *osm-11* may reduce pharyngeal pumping however these changes could be due to differences in outcrossing or stage of animals as

these parameters were not properly monitored. We also screened a RNAi library of ~50 *C. elegans* neuropeptide genes. We rescreened 22 RNAi clones five times (Figure 2B). Most of these RNAi's did not result a reproducible change in pharyngeal pumping, but knockdown of *nlp-12* and *nlp-4* may reduce pharyngeal pumping rate (Figure 2B).

Discussion

Feeding regulatory genes in *C. elegans*

An RNAi screen of 400 genes affecting fat storage revealed that ~30 genes also affect pharyngeal pumping rate or feeding without appreciable effects on development. This suggests that changes in fat storage do not require changes in pharyngeal pumping. In addition, mutations that reduce pharyngeal pumping do not always produce decrease fat storage suggesting that these changes in pumping do not cause the observed fat phenotypes. While pharyngeal pumping is one measure of feeding behavior, other parameters such as foraging rate or time spent on the bacterial lawn may be affected by genes that regulate fat storage in *C. elegans* (Hills, Brockie et al. 2004).

We predicted that *C. elegans* genes regulating both metabolism and feeding behavior may function to convey information about food in the environment. Consistent with this idea, many feeding regulatory genes are expressed in sensory neurons especially the sensory neuron ASI. Earlier chapters discussed the importance of the *daf-7*/TGF- β pathway originating in ASI in the regulation of feeding behavior and fat storage. Interestingly, only four genes *gpd-3*, T21C9.11, Y49A3A.1, and K02E7.11 phenocopy both the Nile red and pharyngeal pumping phenotypes of mutants in the TGF- β pathway. Epistasis analysis suggests that in the presence of dauer pheromone K02E7.11 functions upstream of *daf-7* promoting its expression in ASI (Figure 8A). Consistent with the idea

that feeding regulatory genes may coordinate environmental cues with internal metabolism and feeding, several genes expressed in ASI are regulated by pheromone. Interestingly, genes that are inhibited by pheromone also protect *daf-7* from downregulation by pheromone (Figure 8A). Thus, pheromone may reduce *daf-7::gfp* in part by affecting *daf-7* directly and in part through downregulation of genes that protect *daf-7* (Figure 8A).

Epistasis experiments between the TGF- β ligand *daf-7* and receptor *daf-1* suggest that T05C12.1 functions upstream of the TGF- β pathway to regulate pharyngeal pumping (Figure 8B). While ZK39.7 regulates *daf-7::gfp* in the presence of pheromone, it does not act in the TGF- β pathway to regulate pharyngeal pumping. Preliminary, epistasis experiments suggest that in the presence of pheromone F56H11.6 and K02E7.11 act in the TGF- β pathway. In order to determine if they act in the TGF- β pathway to regulate pharyngeal pumping one could determine if the *daf-3* Co-SMAD suppresses the decreased pharyngeal pumping phenotype of these RNAi animals (Patterson, Kowek et al. 1997). Alternatively, genes expressed in ASI could regulate metabolism and feeding through other pathways in ASI. One candidate is the transcription factor *skn-1*, which functions in ASI to mediate lifespan extension by dietary restriction as has been predicted to respond to internal cues of nutrient availability (Bishop and Guarente 2007).

Epistasis experiments suggest that several feeding regulatory genes modulate pharyngeal pumping through serotonin signaling. *glr-7* and C09G12.5 are expressed in the serotonergic pharyngeal neuron NSM. This suggests they could regulate pharyngeal pumping through regulating the release of serotonin from NSM. *glr-7* encodes for an ionotropic glutamate receptor (Brockie, Madsen et al. 2001), thus glutamate signals

from within the pharynx or other sensory neurons may function upstream of serotonin signaling. Y11D7A.8 and F56H11.6 are epistatic to serotonin signaling and are expressed in the extrapharyngeal nervous system including the ASI neuron. The spatial localization of these genes suggests that there may be two serotonergic pathways affecting feeding behavior. One pathway in the serotonergic pharyngeal neuron NSM is regulated by *glr-7* and C09G12.5 (Figure 8C). A second pathway may function in the extrapharyngeal nervous system, perhaps involving a second source of serotonin, the sensory neuron ADF, and signal through Y11D7.8 and F56H11.6 in ASI (Figure 8C). A similar system may appear in mammals since serotonin functions both in the hypothalamus and enteric neurons and epithelial cells (Tecott 2007). Additional epistasis between feeding regulatory genes and *tph-1* or serotonin GPCR's that regulate feeding, *ser-1* and *ser-7*, will help determine if additional genes function in the serotonergic pathway (Hobson, Hapiak et al. 2005).

Modulation of feeding by starvation

The experience of starvation initially promotes feeding behavior when animals reencounter food. This adaptive response is intact in RNAi animals that have decreased pharyngeal pumping. This data suggests that these animals are capable of fast pumping, but under well-fed conditions limit pharyngeal pumping. In addition, these animals can still respond to chemosensory or mechanosensory aspects of food. Genes that increase feeding when RNAied fail to have a further increase in pharyngeal pumping when reintroduced to food after a period of starvation. This lack of response could be due to limitations on the speed of pharyngeal pumping or indicate that these animals constitutively sense that they have just exited from a starved state. While this modulation

of pharyngeal pumping by starvation and refeeding is interesting we do not understand why these changes occur. In order to understand the assay better we could first determine the role of neurotransmitters serotonin and octopamine which have more defined role in food and starvation sensation (Horvitz, Chalfie et al. 1982; Avery and Horvitz 1990).

Mutants for genes identified by RNAi to affect feeding behavior and fat storage

Deletion mutations in B0310.1, *srd-45*, *cnt-1*, *nkat-1*, and *lag-160* do not have the same phenotype as RNAi of these genes. There are several possibilities for the differences in phenotype for example RNAi could be targeting additional genes or there may be differences in having these genes missing during development rather than only from L1 on. The deletion in *cnt-1* is small and may not fully remove function of the gene explaining why this mutant does not have either a Nile red or pharyngeal pumping phenotype. In *C. elegans* neuronal genes are more resistant to RNAi, thus one possibility for the discrepancy in phenotypes between RNAi and mutant could be due to knockdown of the genes in only a subset of the neurons which express these genes. Understanding the phenotype of these genes is important in order to move forward with additional genetic epistasis.

Table 1: Feeding Regulatory Genes

<i>C. elegans</i> gene ID (predicted function)	Nile Red Phenotype ^a	Feeding Phenotype (% Control)	Human Homolog
Y6B3B.10(<i>tag-160</i> , protein transporter)	Low Nile Red	112 ± 3.1	lag-1 (isoform 1)
F45H7.4(<i>prk-2</i> , kinase)	Low Nile Red	108 ± .5	ser/thr kinase pim-3
F13B6.1 (unknown)	Low Nile Red	112 ± 2.2	membrane mucin (muc17)
Y17G7B.15(<i>cnt-1</i> , Arf GAP)	Low Nile Red	114 ± 3.8	centaurin-beta
C02F4.2 (<i>tax-6</i> , calcineurin α)	Low Nile Red	114 ± 3.2	phosphatase 2B
ZK39.7(C-type lectin,)	Low Nile Red	111 ± 2.6	chondrotin sulfate proteoglycan
T05C12.1(casein kinase)	Low Nile Red	113 ± 3.1	tau tubulin kinase
F28H6.3 (<i>nkat-1</i> , kynurenine aminotransferase III)	Low Nile Red	119 ± .7	KAT3
F17A2.7 (<i>srd-45</i> chemosensory receptor)	Low Nile Red	113 ± 1.8	GPCR
B0310.1(potassium channel)	Low Nile Red	112 ± 5.4	potassium channel
K10B3.7 (<i>gpd-3</i> , GAPDHase-2)	Low Nile Red	86 ± 4.7	GAP Dehydrogenase
T21C9.11 (unknown)	Low Nile Red	85 ± 2.3	N/S (no significant homology)
Y49A3A.1 (phospholipid biosynthesis)	Low Nile Red	93 ± 3.1	choline/ethanolamine phosphotransferase
K02E7.11 (unknown)	Low Nile Red	87 ± 2.0	lag-1 homolog
Y11D7A.8 (kinase)	High Nile Red	78 ± 9.4	N/S
Y46H3C.1 (rhodopsin-like GPCR <i>srw-100</i>)	High Nile Red	79 ± 1.0	GPCR
F56H11.6 (casein kinase)	High Nile Red	90 ± 1.4	tau tubulin kinase
C43H6.9 (<i>glr-7</i> , glutamate receptor)	High Nile Red	84 ± 3.5	GluR 6
C09G12.5 (unknown)	High Nile Red	92 ± 2.1	N/S
C43H6.8 (<i>hlh-15</i> , transcription factor)	High Nile Red	85 ± 4.0	hlh-2
F25H8.5 (<i>dur-1</i>)	High Nile Red	82 ± 1.9	ribosome binding protein
F31F6.2 (unknown)	High Nile Red	81 ± 3.2	N/S
F26H9.4 (involved in biosynthesis of NAD)	High Nile Red	110 ± 1.9	Nicotinamide adenylyl transferase

^a Nile red phenotype defined in Ashrafi. et. al (2003)^b Average of % Vector Control pumping for 5 independent experiments

Table 2: Mutant phenotype for genes identified by RNAi to affect nile red and pharyngeal pumping

Gene	RNAi phenotype ^a	Mutant (# backcrosses)	Mutant Phenotype
Y6B3B.10	increased pumping, reduced nile red	<i>tag-160</i> (2X)	Reduced pumping ^b
<i>prk-2</i>	increased pumping, reduced nile red	<i>prk-2(pk278)</i> (4X)	Increased pumping (variable), no nile red phenotype
B0310.1	increased pumping, reduced nile red	B0310.1(tm2480) (3X)	decreased pumping, increased nile red, little short; fails to suppress <i>daf-7</i>
<i>srd-45</i>	increased pumping, reduced nile red	<i>srd-45(tm2196)</i> (4X)	increased nile red, no pumping phenotype. Fails to suppress <i>tph-1</i> or <i>daf-7</i>
<i>cnt-1</i>	increased pumping, reduced nile red	<i>cnt-1(tm2313)</i> (4X)	none; fails to suppress <i>tph-1</i> or <i>daf-7</i>
<i>dur-1</i>	decreased pumping; increased nile red	<i>dur-1(ok1010)</i> (0X)	decreased pumping
<i>srw-100</i>	decreased pumping; increased nile red	<i>srw-100(tm2540)</i> (2X)	decreased pumping; increased nile red
F28H6.3	increased pumping, reduced nile red	<i>nkcat-1(ok566)</i> (2X)	reduced pumping ^b
<i>tax-6</i>	increased pumping, reduced nile red	<i>tax-6(p675)</i>	reduced pumping

^a Nile Red phenotype from Ashrafi. et al (2003)

^b Personal communication A. Kahn-Kirby

Figure 1: Genes regulating pharyngeal pumping and Nile red staining in *C. elegans*

(A) RNAi inactivation of 23 genes previously identified to affect Nile red staining also affect pharyngeal pumping rates. Pharyngeal pumping rates are 10-20% above or below the rate of animals on control RNAi. Pharyngeal pumping data is an average of % vector control for five replicates. Error bars indicate standard deviation in % vector control for each RNAi tested. Nile red phenotypes obtained from Ashrafi. et. al (2003).

(B) RNAi inactivation of 5 genes results in decreased pharyngeal pumping. This decrease in pharyngeal pumping is likely to be due to developmental abnormalities. Pharyngeal pumping data is an average of % vector control for five replicates. Error bars indicate standard deviation in % vector control for each RNAi tested. Nile red phenotypes obtained from Ashrafi. et. al (2003).

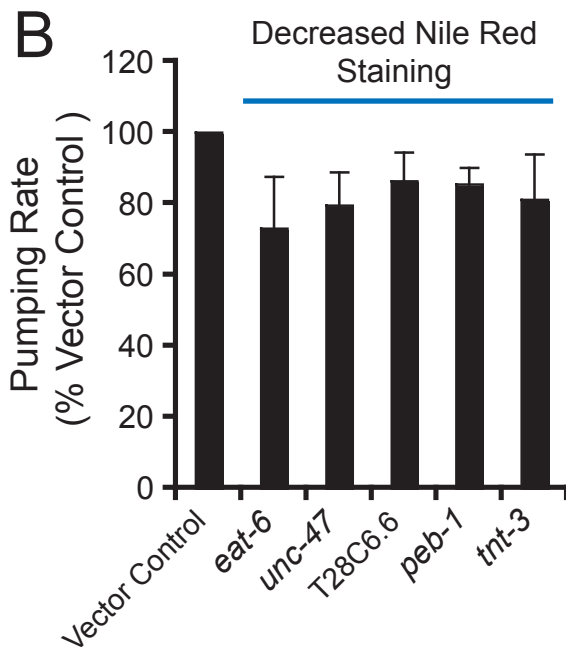
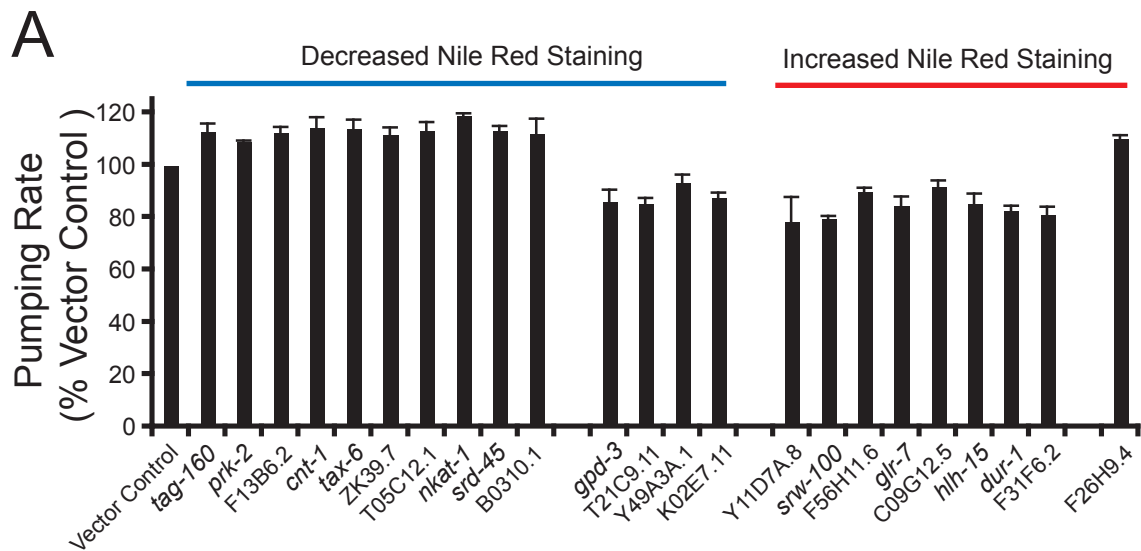


Figure 2: Expression pattern of feeding regulatory genes

(A) Several feeding regulatory genes are expressed in pharyngeal muscle. Transcriptional (*tag-160*, *prk-2*, and *cnt-1*) and translational (F13B6.1) GFP fusions using ~2kb of promoter show expression in pharyngeal muscle. *pprk-2::gfp* and *pcnt-1::gfp* have additional expression in the nervous system.

(B) Neuronal expression of feeding regulatory genes. ZK39.7, T05C12.1, *nkat-1*, *srw-100*, and F56H11.6 GFP reporter fusions are expressed exclusively in the sensory neuron ASI indicated by white arrowheads. Translational GFP reporters for *gpd-3* and T21C9.11 show broad neuronal expression. Y49A3A.1 and K02E7.11 GFP reporters are expressed in ASI in addition to unidentified neurons and pharyngeal neurons NSM and M1 respectively. Y11D7A.8 is expressed in labial neurons in addition to dim expression in ASI. C09G12.5 is expressed in the pharyngeal neuron NSM indicated by white arrowhead. F26H9.4 is expressed in the sensory neuron ADL (indicated by white arrowhead) as well as unidentified labila neurons.

(C) Translational fusion of GFP to B0310.1 genomic fragment and transcriptional GFP fusion of *dur-1* both show expression in the hypodermis.

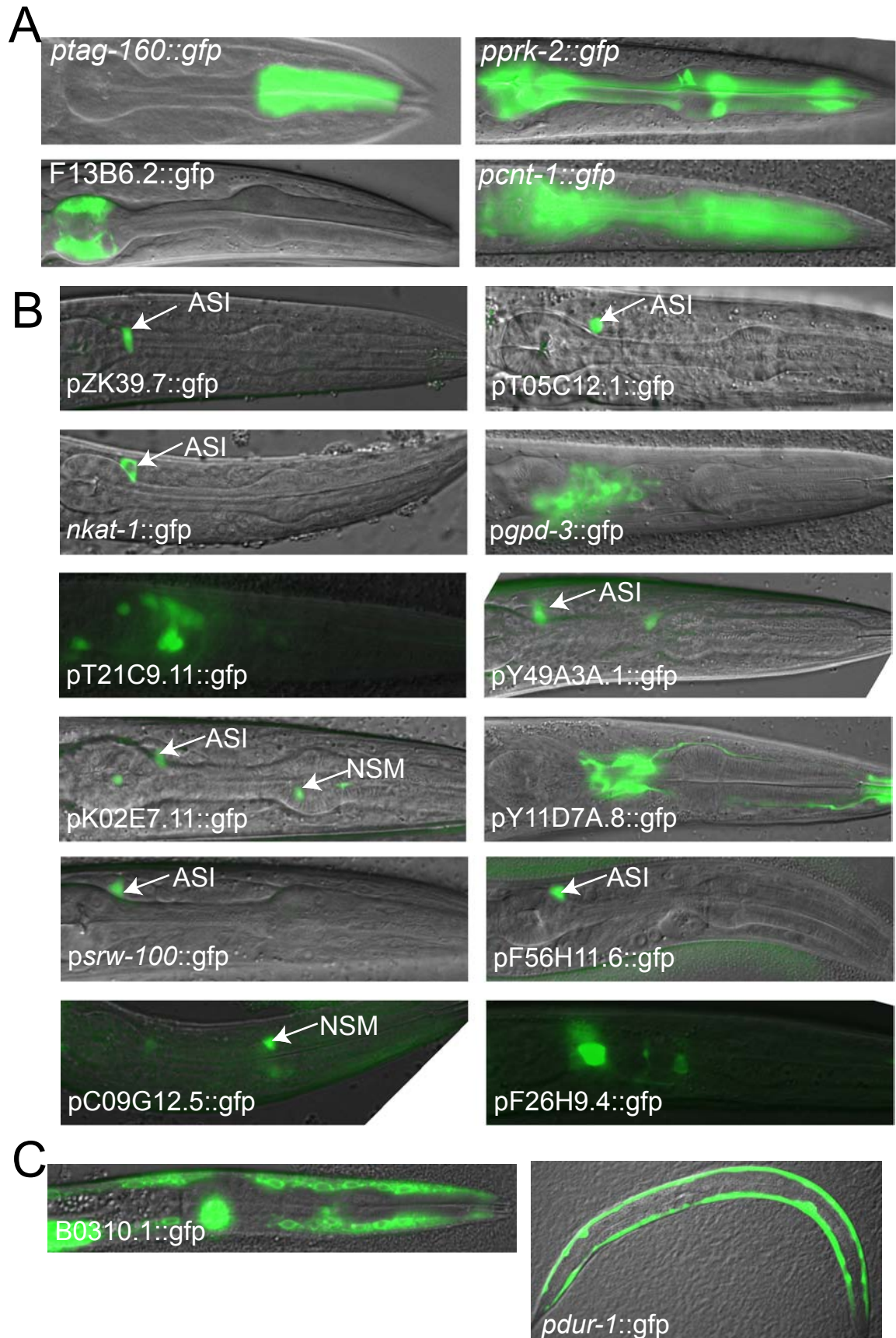


Figure 3: Feeding regulatory genes are regulated by pheromone and modulate *daf-7::gfp* response to pheromone.

(A) Addition of crude extract of *C. elegans* dauer pheromone downregulates expression of *daf-7::gfp* in ASI. Similarly expression of pZK39.7::gfp and pT05C12.1::gfp in ASI decreases in intensity with addition of dauer pheromone. Expression of *psrw-100::gfp* in ASI is increased by addition of dauer pheromone. White arrowheads indicate position of ASI neuron.

(B) Feeding regulatory genes modulate response of *daf-7::gfp* to dauer pheromone. Pheromone downregulates *daf-7::gfp* expression in ASI. This effect is exacerbated when ZK39.7, T05C12.1, or K02E7.11 are knocked down by RNAi in the presence of pheromone. White arrowheads indicate position of ASI neuron.

(C) RNAi of B0310.1 or *dur-1* increases *daf-7::gfp* expression in response to pheromone treatment. White arrowheads indicate position of ASI neuron.

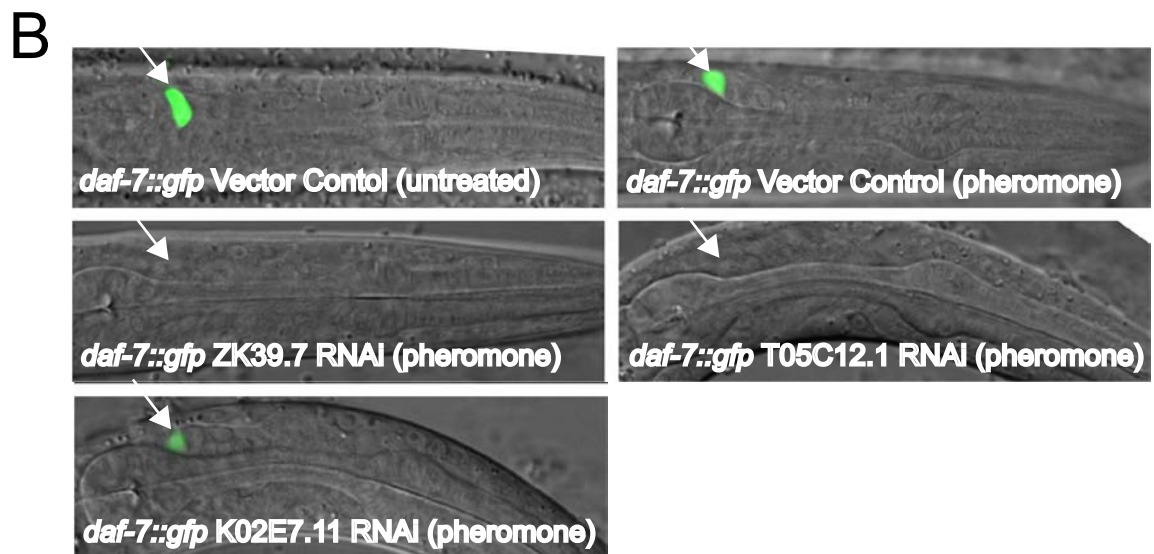
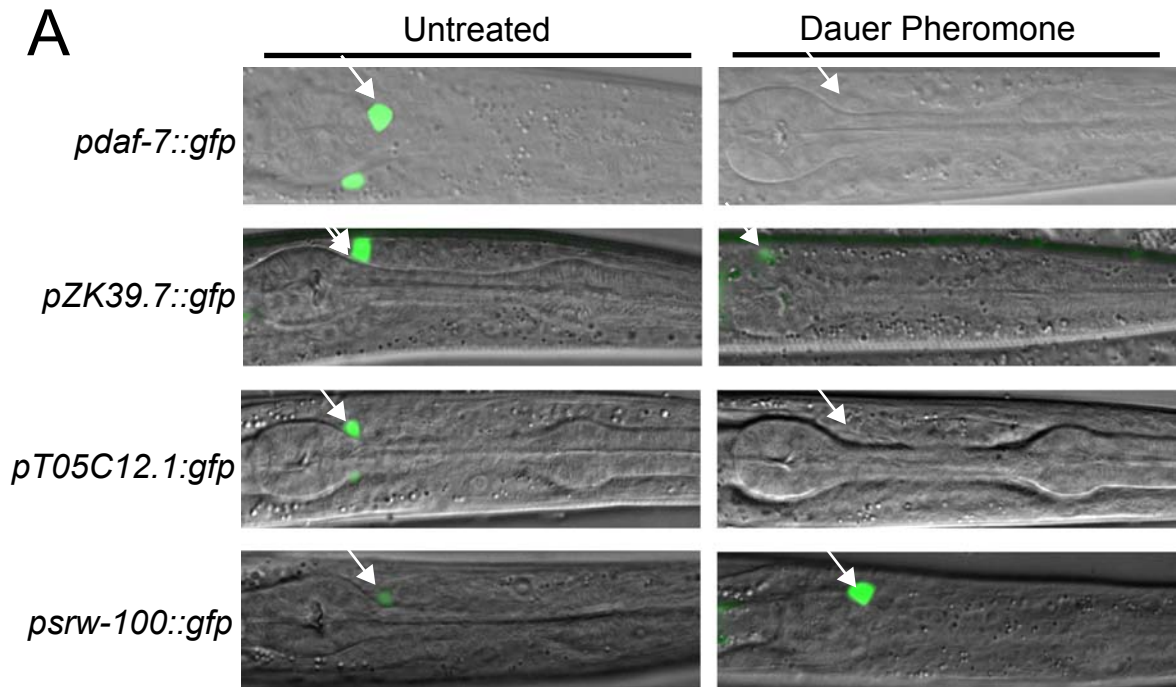


Figure 4: Feeding regulatory genes interact with TGF- β , glutamate, and serotonin signaling to regulate pharyngeal pumping.

(A) Each of ten genes that increase pharyngeal pumping were RNAi'd in *daf-1(m40)* and *daf-7(e1372)* animals. Mutations in *daf-1* and *daf-7* reduced pharyngeal pumping to 83% and 78% of WT. With the exception of T05C12.1, RNAi of genes that increased pharyngeal pumping rate increased the pharyngeal pumping rate of *daf-1(m40)* and *daf-7(e1372)* animals. Data represented is the summary of four independent experiments. Standard deviation bars are indicated.

(B) Mutants in the vesicular glutamate transporter, *eat-4*, have pumping rate that is 80% of WT. Genes that increase pharyngeal pumping were RNAi'd in *eat-4(ad819)* animals. RNAi of F13B6.2, ZK39.7, and *srd-45* failed to increase the pumping rate of *eat-4(ad819)* animals. Data represented is the summary of five independent experiments. Standard deviation bars are indicated.

(C) Addition of 5mM serotonin increases pharyngeal pumping by 10%. RNAi of T21C9.11, *glr-7*, and C09G12.5 decrease pharyngeal pumping (79%, 78%, and 83% of control) however this effect is abolished in the presence of 5mM serotonin. Likewise, RNAi of Y11D7A.8 and F56H11.6 also reduce pharyngeal pumping (79% and 87% of control) yet, suppress the increased pharyngeal pumping of 5mM serotonin.

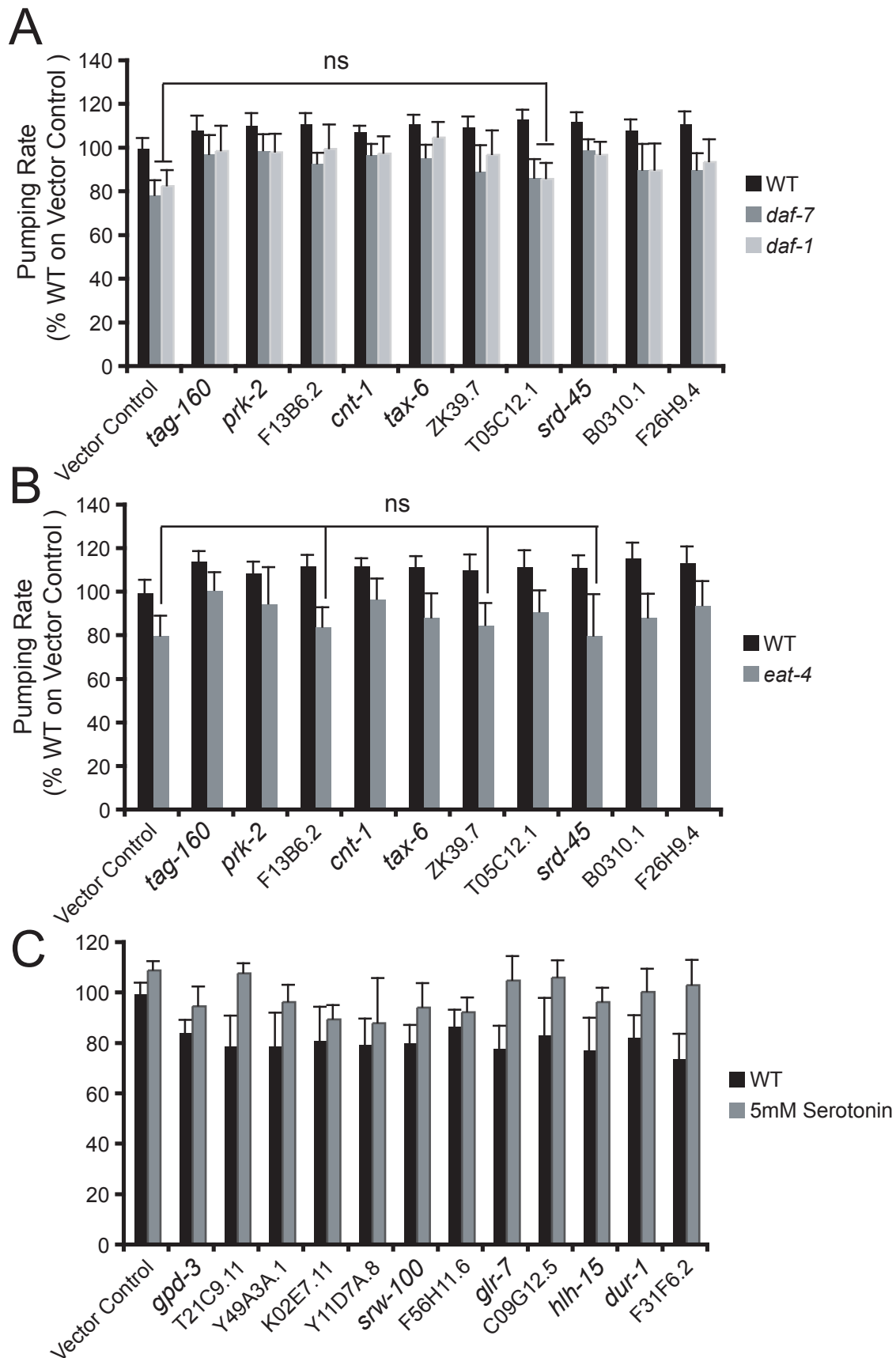


Figure 5: Response of feeding regulatory genes to starvation and refeeding.

(A-B) Pharyngeal pumping rates are assayed immediately as animals were returned to food after either 0 hours (blue bars), 2 hours (red bars) or 5 hours (yellow bars) of starvation in liquid media. Pharyngeal pumping rates were also assayed as animals recovered from a 5 hour starvation for either 1 hour (green bars) or 3 hours (purple bars). Standard deviation bars are shown for 1-3 replicates for each RNAi shown.

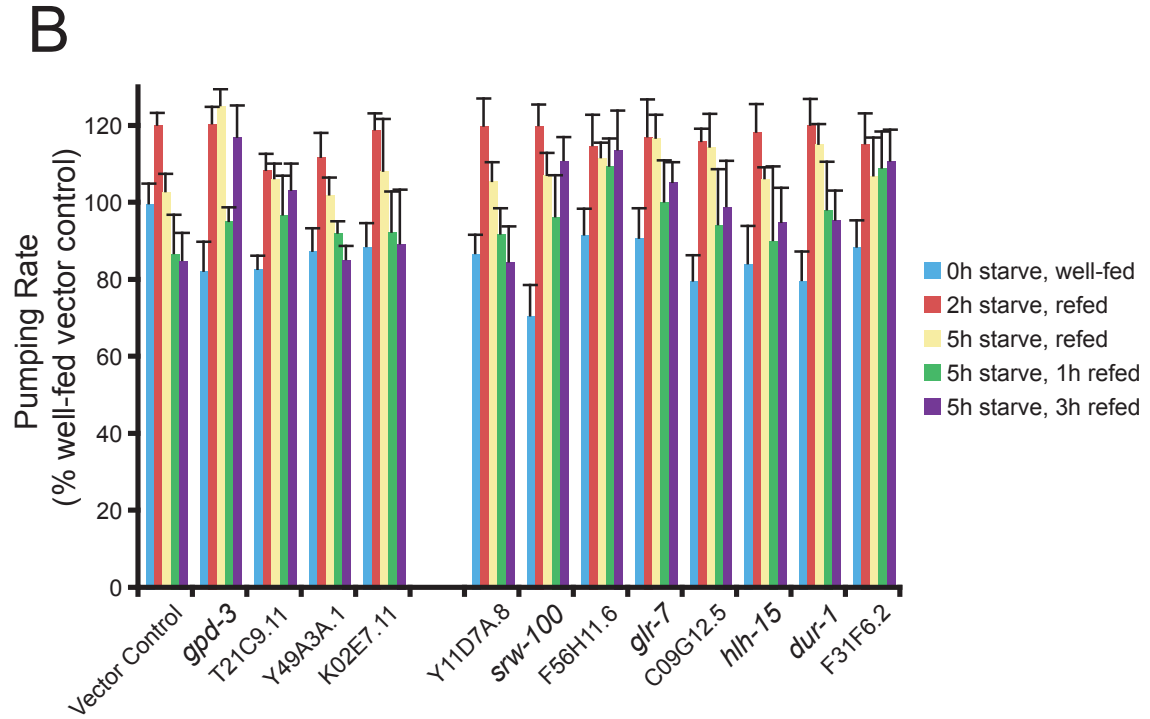
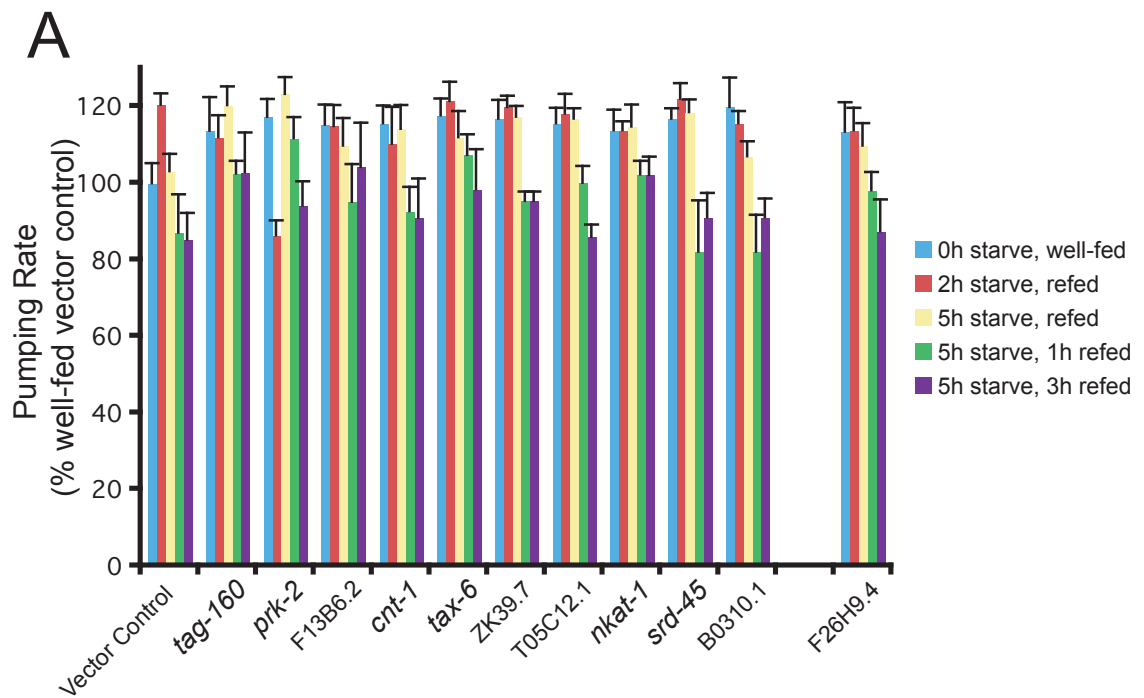


Figure 6: Pharyngeal pumping and Nile red staining of mutants in feeding regulatory genes

(A) Mutations in *tax-6*, *dur-1*, *srw-100*, and B0310.1 cause a reduction in pharyngeal pumping rates compared to wild type. Error bars indicate standard deviation.

(B) Mutations in *srd-45* and *cnt-1* do not produce a pharyngeal pumping phenotype and fail to suppress the reduction of pumping rate in *tph-1* serotonin deficient animals. Error bars indicate standard deviation.

(C) *prk-2* mutants have a slight increase in pharyngeal pumping rate and mildly suppress the decreased pharyngeal pumping phenotype of *daf-1* animals. *cnt-1* mutant animals do not suppress the decreased pharyngeal pumping phenotype of *daf-7* mutants.

(D) Examples of nile red staining for WT, *prk-2*, *srd-45*, and B0310.1 mutants. *prk-2(pk278)* animals have similar nile red staining as wild type. Both *srd-45(tm2196)* and B0310.1(tm2480) have increased nile red staining intensity.

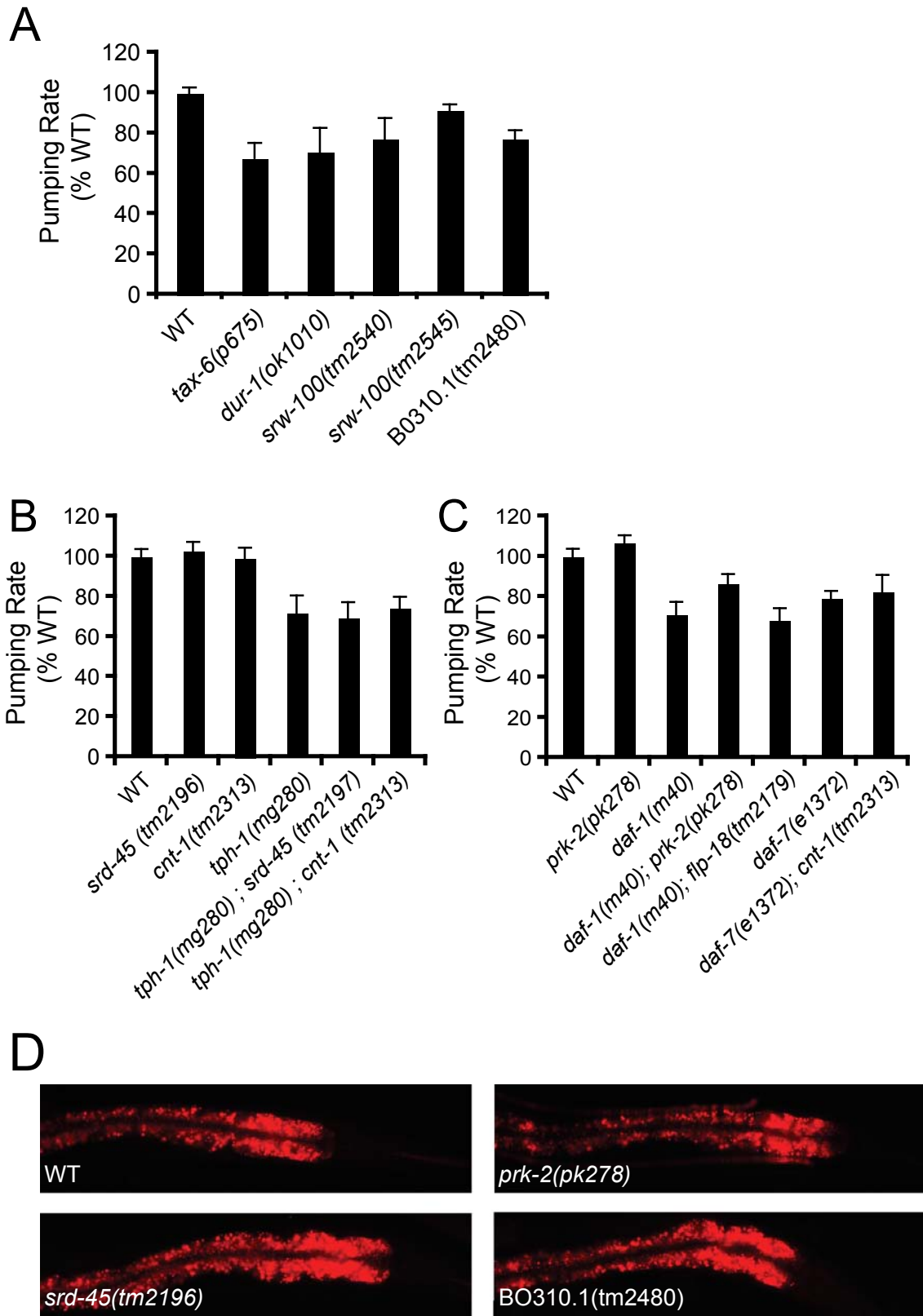


Figure 7: Additional genes may regulate pharyngeal pumping

- (A) Mutations in some chemosensory genes may alter pharyngeal pumping rates, however animals may not have been appropriately staged with respect to wild type at the time pharyngeal pumping was assayed.
- (B) RNAi of many neuropeptides does not alter pharyngeal pumping rate compared to vector control animals. RNAi of *nlp-12*, *nlp-4* may reduce pharyngeal pumping rate. Graph depicts average of % vector control pumping rate from 5 independent experiments. Error bars indicate standard deviation.

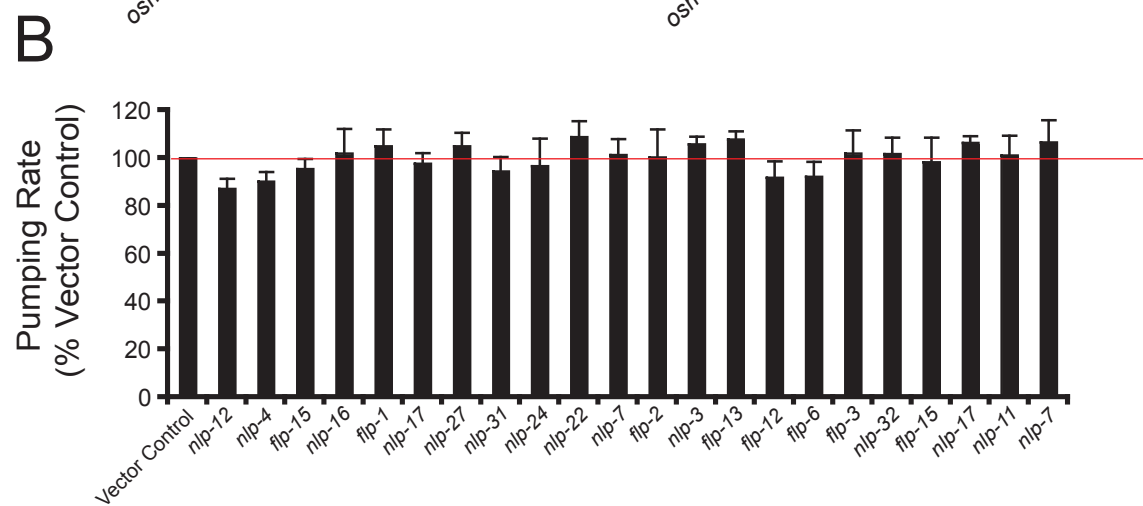
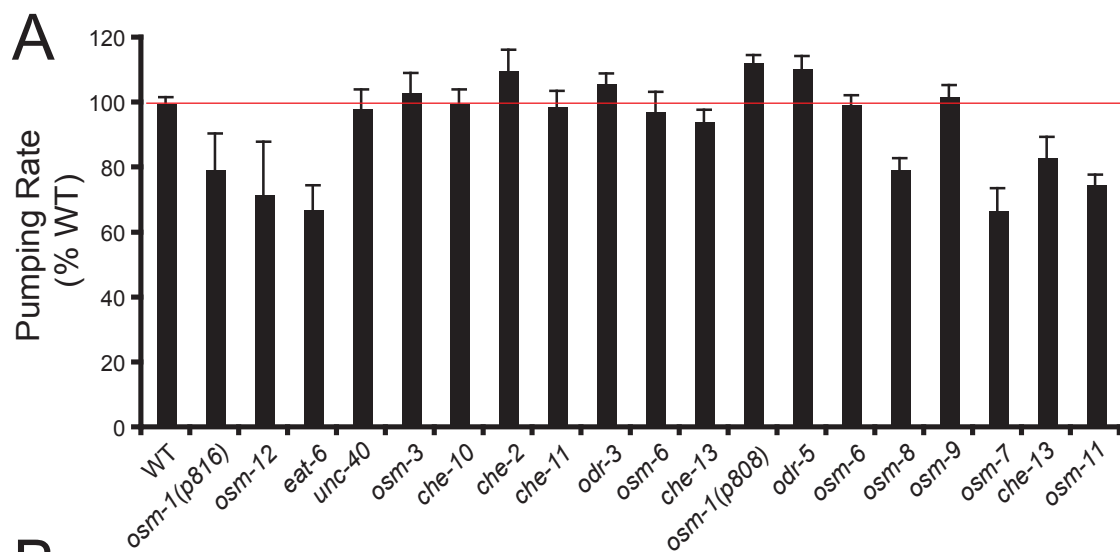


Figure 8: Models for interaction between feeding regulatory genes and TGF- β and serotonin signaling.

(A) High levels of dauer pheromone inhibit ASI neuronal expression of ZK39.7, T05C12.1, K02E7.11, F56H11.6, and *daf-7*. In the presence of pheromone, loss of ZK39.7, T05C12.1, K02E7.11, and F56H11.6 further reduce *daf-7* expression suggesting they normally protect *daf-7*. While B0310.1 and *dur-1* which are expressed in the hypodermis inhibit *daf-7* expression.

(B) In conditions where dauer pheromone concentration is low the kinase T05C12.5 regulates pharyngeal pumping through DAF-7. Similarly, K02E7.11 and F56H11.6 may also act upstream of DAF-7 however this has not been tested in low pheromone conditions

(C) C09G12.5 and *glr-7* are expressed in the serotonergic NSM pharyngeal neurons and regulate pharyngeal pumping through serotonin signaling suggesting they may modulate serotonin release from NSM. Y11D7A.8 and F56H11.6 are expressed in extrapharyngeal neurons including ASI and suppress the effects of serotonin on pharyngeal pumping. These genes may function in a separate serotonergic pathway. Serotonin from the ADF neuron may signal to Y11D7A.8 and F56H11.6 in ASI to regulate pharyngeal pumping.

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Chapter VII: Conclusions and future directions

Key Conclusions

TGF- β Signaling regulates pharyngeal pumping, fat storage, and dauer formation

The integration of neural and genetic circuit data presented here leads to a complex model for TGF- β regulation of feeding behavior, fat storage, and dauer formation. The TGF- β ligand *daf-7* is expressed in the sensory neurons ASI (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996). *daf-7* acts as a monitor of environmental conditions such as food availability and population density. Increasing population density leads to increased concentration of dauer pheromone, which results in downregulation of *daf-7* transcription (Ren, Lim et al. 1996). Similarly, adverse conditions such as reduced food availability reduce *daf-7* transcription (Ren, Lim et al. 1996). Here we show that *daf-7* functions as a signal of environmental conditions both in development and adulthood.

Neural circuit analysis reveals that DAF-7 signals to the type I TGF- β receptor DAF-1 in the RIM and RIC interneurons to regulate feeding behavior, fat storage, and dauer formation. Activation of DAF-1 in these neurons leads to inhibition of the co-SMAD DAF-3. This limited neural circuit highlights the importance of coordinate regulation of feeding, fat storage, and dauer.

Genetic epistasis reveals that downstream of the co-SMAD *daf-3* distinct signals from RIM and RIC independently regulate pharyngeal pumping, fat storage, and dauer formation. RIM and RIC are the site of tyramine and octopamine synthesis in *C. elegans*. Active TGF- β signaling in these neurons decreased tyramine and octopamine signaling to modulate pharyngeal pumping. Dauer entry but not fat storage or pharyngeal pumping is

regulated by the nuclear hormone receptor DAF-12. Similarly, metabotropic glutamate receptors signaling through G-proteins modulate fat storage but not dauer formation or pharyngeal pumping.

Signals regulating fat storage downstream of TGF- β are likely to be distributed. We identified two metabotropic glutamate receptors, *mgl-1* and *mgl-3*, which function in parallel to increase fat storage. Mutations in both *mgl-1* and *mgl-3* only partially suppress the fat storage phenotype of TGF- β mutants, suggesting that there may be additional signaling pathways that regulate fat storage. Furthermore, the neural circuit downstream of RIM and RIC regulating fat storage is also likely to be widely distributed. *mgl-1* and *mgl-3* are expressed in several non-overlapping head neurons. Since these receptors act in parallel, it is likely that they function in different sets of neurons to regulate fat storage.

Together these data suggest the existence of a complex genetic and neural circuitry allowing the TGF- β pathway to coordinate multiple aspects of *C. elegans* physiology with environmental conditions.

Additional genes regulate feeding behavior

We identified ~20 additional genes that affect pharyngeal pumping and fat storage as measured by accumulation of Nile red. Several of these genes are expressed in the pharyngeal muscle and neurons, suggesting that they act locally within the pharynx to modulate pharyngeal pumping. Many of these genes are expressed in the ASI sensory neuron, emphasizing the importance of this neuron in coordinating metabolism and pharyngeal pumping. These genes may interact with other key pathways regulating

pharyngeal pumping and fat storage, including the TGF- β and serotonin signaling pathways.

Future Directions

Targets of TGF- β signaling in RIM and RIC

Active TGF- β signaling leads to the nuclear accumulation of transcription factors called SMADs. In *C. elegans*, DAF-8 and DAF-14 SMADs function downstream of the TGF- β pathway by inhibiting the co-SMAD DAF-3. As transcription factors, SMADs are thought to function by eliciting changes in gene expression. Recent microarray studies examining changes in gene expression show that ~5000 genes are upregulated or downregulated when comparing adult wild type and TGF- β pathway mutants (Shaw, Luo et al. 2007). This large set includes genes involved in growth, development, metabolism, behavior, and a wide range of cellular processes (Shaw, Luo et al. 2007). While these experiments illustrate that changes in TGF- β signaling result in significant alterations in gene expression, the large number of genes identified makes it difficult to focus on specific genes or signaling pathways to investigate further. In addition, these experiments used all RNA extracted from whole animals; thus, it is unclear if these genes are direct or indirect targets of SMAD transcription factors.

We determined that the co-SMAD DAF-3 functions in RIM and RIC interneurons to regulate pharyngeal pumping, fat storage, and dauer formation. Changes in gene expression in RIM and RIC could be analyzed in order to examine more specific effects of TGF- β signaling in cells where the TGF- β pathway is actively signaling. Gene expression changes in single neurons can be assayed by expressing an epitope-tagged polyA binding protein (FLAG::PAB-1) in specific neurons (Von Stetina, Watson et al. 2007). We can then immunoprecipitate PAB-1 using the epitope tag and isolate mRNA

bound by PAB-1 (Von Stetina, Watson et al. 2007). Again, we can take advantage of the *tdc-1* and *tbh-1* promoters, which respectively drive expression in RIM, RIC, and UV1cells and RIM (Alkema, Hunter-Ensor et al. 2005). Using these promoters to drive FLAG::PAB-1 in RIM and RIC we can isolate RNA specifically from these neurons. RNA isolated in this manner can be examined by microarray for changes in gene expression in adult wild type and TGF- β mutants.

We still do not understand which signals from RIM or RIC lead to changes in steroid hormones to regulate dauer formation or glutamate signaling to regulate fat storage. Thus, changes in gene expression of secreted or signaling factors should be a high priority area of study. By selectively examining gene expression changes in RIM and RIC we can focus on transcriptional changes in cells of interest.

Additional signals regulating fat storage in the TGF- β pathway

Loss of glutamate signaling partially suppresses the fat storage phenotype of *daf-7* TGF- β ligand mutants, suggesting that additional factors may also regulate fat storage. We discovered that alterations in G-protein signaling, including loss of function in G_o signaling or gain of function in G_q signaling, suppressed fat accumulation of *daf-7* animals more completely. One possibility is that additional unidentified GPCR's could function downstream of TGF- β signaling. Alterations in G_o and G_q signaling also result in hyperactivity, which could also reduce the fat content of *daf-7* mutants by increasing energy expenditure in locomotion. It is clear that additional genes function downstream of TGF- β signaling to regulate fat storage; however, it is not clear that these genes are GPCR's.

Additional genetic suppressor screening could identify genes functioning downstream of TGF- β signaling to regulate fat storage. In the screen we performed, we only identified one gain of function allele of *egl-30* and failed to identify alleles of *dgk-1* and *goa-1*, although these genes also suppress the fat accumulation of *daf-7* mutant animals. This suggests that the screen did not reach saturation, and further screening could be productive. One caveat to this approach is that weak suppressors of *daf-7(e1372)* such as *mgl-1* and *mgl-3* may not be identified. Alternatively, genes identified in cell-specific array studies suggested above, or genes identified in other TGF- β suppressor screens, could also be examined for their role in regulating fat storage.

The nuclear hormone receptor DAF-12 suppresses the dauer formation phenotype of mutants in the insulin and TGF- β pathways. We establish that DAF-12 does not regulate fat storage in the TGF- β pathway. While this suggests that dauer formation and fat storage phenotypes are separable, it is possible that signaling upstream of DAF-12 that is specific to the TGF- β pathway will function in both dauer formation and fat storage.

Inoue and Thomas identified several genes that suppress dauer formation of TGF- β mutants but not insulin pathway mutants (Inoue and Thomas 2000). They identified three novel genes, *scd-1*, *scd-2*, and *scd-3*, as partial suppressors of the dauer constitutive phenotype of TGF- β mutants (Inoue and Thomas 2000). *scd-1* partially suppresses other phenotypes of TGF- β mutants such as dark color of the intestine, egg-laying, and bordering the edge of the bacterial lawn (Inoue and Thomas 2000). In contrast, *scd-2* and *scd-3* only suppress the dauer formation and bordering phenotypes of TGF- β mutants (Inoue and Thomas 2000). *scd-3* remains uncloned, but *scd-1* encodes a glutamine rich protein of unknown function and *scd-2* encodes a protein tyrosine kinase

(www.wormbase.org). Both *scd-1* and *scd-2* are broadly expressed in neurons, intestine, and body wall (www.wormbase.org). The role of these genes in fat storage is unknown, but examining this area may reveal a more complex relationship between fat storage and dauer formation.

Role of tyramine in pharyngeal neurons

In these studies we show that expression of the tyramine GPCR *ser-2* regulates pharyngeal pumping in pharyngeal neurons downstream of TGF- β signaling. GFP reporter analysis shows that *ser-2* is expressed in the pharyngeal neuron NSM (Tsalik, Niacaris et al. 2003). Our analysis reveals that expression of *ser-2* under the *glr-7* promoter, which drives expression in several pharyngeal neurons including NSM, rescues *ser-2* mutant phenotypes. Together these data suggest that *ser-2* may function in NSM to regulate pharyngeal pumping. This could be tested using promoters specific for NSM (Zhang, Lu et al. 2005).

In addition, neurotransmitters or neuropeptides are likely to communicate signals from pharyngeal neurons expressing *ser-2* to pharyngeal muscle in order to alter pharyngeal pumping rate. Several key neurotransmitters in the pharyngeal neurons are acetylcholine, glutamate, and serotonin (Avery and Thomas 1997). NSM is thought to secrete both glutamate and serotonin neurotransmitters; thus, *ser-2* may impinge on these signaling systems to regulate pharyngeal pumping downstream of TGF- β signaling (Avery and Thomas 1997; Lee, Sawin et al. 1999).

Octopamine receptors in the TGF- β pathway

Both tyramine and octopamine regulate pharyngeal pumping in the TGF- β pathway. We find that the tyramine GPCR *ser-2* mediates the effects of TGF- β signaling

on pharyngeal pumping, but we do not know which octopamine GPCR plays a similar role. Until recently, *ser-3* was the only identified octopamine receptor in *C. elegans* (Suo, Kimura et al. 2006). We found that *ser-3* mutants have a decreased pharyngeal pumping rate; thus, this receptor cannot suppress the decrease in pharyngeal pumping of TGF- β mutants. Recently, researchers identified F14D12.6 as an additional *C. elegans* octopamine receptor and bioinformatics data from their study suggest that Y54G2A.35 is also an octopamine receptor (Wragg, Hapiak et al. 2007). One of these receptors, or an unidentified octopamine receptor, may function downstream of TGF- β signaling to regulate pharyngeal pumping. We hypothesize that identifying both the tyramine and octopamine receptors that regulate pharyngeal pumping in the TGF- β pathways will result in a full suppression of the pumping defects of *daf-1* and *daf-7* mutant pumping.

Possible additional metabolic and behavioral defects in TGF- β mutants

Collaborators in the Van Gilst lab determined that alterations in TGF- β signaling lead to increased *de novo* fat synthesis. TGF- β mutants may have increased fat stores as a result of this increase in fat synthesis. TGF- β mutants could also have defects in metabolic rate or energy expenditure, which can be monitored by assaying the rate of oxygen consumption. Since TGF- β mutants have increased fat stores, they may have reduced energy expenditure, which will manifest as reduced oxygen consumption rates.

RNAi screens could identify additional metabolic genes in the TGF- β pathway. By treating *daf-1* or *daf-7* with RNAi to knock down metabolic genes including β -oxidation (fat breakdown), fat synthesis, and fatty acid binding proteins, we could define specific metabolic genes that function downstream of TGF- β signaling to regulate fat storage. This approach has been successful in identifying a specific metabolic pathway

that results in a reduction in fat stores when animals are treated with exogenous serotonin (personal communication, S. Srinivasan). Using this RNAi based approach is preferable over mutagenesis, since many metabolic genes may be lethal or cause developmental abnormalities when mutated. RNAi allows us to circumvent possible developmental delay or lethality associated with loss of metabolic genes.

The experiments above will address the roles of metabolic changes in the excess fat accumulation of TGF- β mutants. In addition to metabolic changes, behavioral adaptations may also play a role in fat storage. In this work we show that although TGF- β mutants have defects in feeding behavior or pharyngeal pumping, these changes in pharyngeal pumping do not cause excess fat accumulation. Other behaviors such as rate of locomotion or time spent on the bacterial lawn (food source) could cause changes in fat storage; however, these behaviors have not been examined in TGF- β mutants.

In addition, energy stores can be used for reproduction. Animals that are unable to accumulate fat stores appropriately often have reduced brood size (Yang, Vought et al. 2006). Mutants in the TGF- β pathway fail to lay eggs at a normal rate, leading to an accumulation of eggs in the uterus (Trent, Tsuing et al. 1983). It is unclear if this alters the brood size produced by TGF- β mutants. Furthermore, we do not know whether more energy is used in egg-laying or egg production. Understanding these metabolic and behavioral parameters will lead to a better understanding of how the TGF- β pathway regulates metabolism and how *C. elegans* maintains energy homeostasis.

Interaction between feeding regulatory genes and the TGF- β pathway

Using an RNAi based approach, we identified ~20 genes that alter both feeding rate (pharyngeal pumping) and fat storage (accumulation of Nile red). Of these, we

identified 7 genes that are expressed almost exclusively in the sensory neuron ASI. This neuron releases the TGF- β ligand *daf-7*. Several environmental factors affect the level of *daf-7* expression in ASI. Dauer pheromone, which signals a high population density, reduces *daf-7* expression, while abundant food promotes *daf-7* expression. In order to determine if these genes interact with the TGF- β pathway, we determined if dauer pheromone regulated expression of these genes and if they altered the response of *daf-7* to dauer pheromone. These preliminary experiments suggested that dauer pheromone regulates the expression of ZK39.7, T05C12.1, K02E7.1, and F56H11.6. Furthermore, these four genes alter the response of *daf-7* to dauer pheromone. B0310.1 and *dur-1*, which are expressed in the hypodermis, also alter the response of *daf-7* to dauer pheromone. Epistasis analysis revealed that only T05C12.1 functions upstream of TGF- β signaling to regulate pharyngeal pumping. However, the epistatic relationship between the TGF- β pathway and K02E7.11, F56H11.6, and *dur-1* could not be established, since RNAi of these genes also reduces pharyngeal pumping. Mutations in *daf-3*, *tdc-1*, *tbh-1*, or *ser-2* suppress the reduced pharyngeal pumping of *daf-1* and *daf-7* mutants. Thus, in order to determine if K02E7.11, F56H11.6, and *dur-1* interact with the TGF- β pathway, we could determine if mutations *daf-3*, *tdc-1*, *tbh-1*, or *ser-2* suppress the pumping phenotypes of RNAi of these genes.

The interaction of feeding regulatory genes with the TGF- β pathway should also be examined for fat storage. Epistasis analysis between genes that reduce fat storage and high fat *daf-7* or *daf-1* mutants could be performed. Genes that increase fat storage may be suppressed by mutations in *daf-3* or *eat-4*, both of which suppress the fat storage phenotypes of *daf-7* or *daf-1* mutants. In this manner, we can determine if additional

genes also regulate pharyngeal pumping and fat storage in the paradigm determined for the TGF- β pathway.

Interaction between feeding regulatory genes and serotonin signaling

Serotonin signaling also regulates fat storage and pharyngeal pumping in *C. elegans* (Segalat, Elkes et al. 1995; Sze, Victor et al. 2000). Serotonin is produced by tryptophan hydroxylase, *tph-1*. Mutations in *tph-1* result in increased fat storage, reduced pharyngeal pumping, and increased dauer formation (Sze, Victor et al. 2000). *tph-1* is expressed in serotonergic neurons, including the pharyngeal neurons NSM, sensory neurons ADF, and hermaphrodite-specific neuron HSN. In contrast, addition of exogenous serotonin leads to increased pharyngeal pumping and decreased fat storage (personal communication, S. Srinivasan).

The *tph-1* mutant and exogenous serotonin provide us with tools we can use to determine the interactions between feeding regulatory genes and serotonin signaling. Using exogenous serotonin and assaying for pharyngeal pumping, we have determined that C09G12.5 and *glr-7* act upstream of serotonin signaling. Both of these genes are expressed in the pharyngeal serotonergic neuron NSM. It is possible that RNAi of these genes could alter the expression of *tph-1::gfp* reporter in NSM, suggesting that these genes function by regulating serotonin release from NSM. In addition, we found that Y11D7A.8 and F56H11.6, which are expressed in the nervous system, including the sensory neuron ASI, function downstream of serotonin to regulate pharyngeal pumping. This suggests that two pathways, one in the pharyngeal nervous system and one in the extrapharyngeal nervous system, may regulate pharyngeal pumping. Additional epistasis experiments with *tph-1* will determine if other feeding regulatory genes interact with

serotonin signaling. In addition, it is important to determine if the epistatic interactions described above are specific for pharyngeal pumping, or if these genes also function in the serotonergic pathway leading to alterations in fat storage.

Genes that interact with serotonin signaling to regulate fat storage or pharyngeal pumping can also be examined for interactions with specific serotonergic receptors. Two serotonergic GPCR's, *ser-1* and *ser-7*, mediate the effects of exogenous serotonin on pharyngeal pumping (Hobson, Hapiak et al. 2005; Dernovici, Tanja et al. 2007). However, these receptors do not abrogate the effects of exogenous serotonin on fat storage (personal communication, S. Srinivasan). Similarly, the serotonin-gated chloride channel *mod-1* (Ranganathan, Cannon et al. 2000) suppresses the effects of exogenous serotonin on fat storage but not pharyngeal pumping (personal communication, S. Srinivasan). Epistasis experiments with *tph-1*, *mod-1*, *ser-1*, and *ser-7* will lead to a better understanding of the feeding regulatory genes in the context of serotonin signaling.

Concluding Remarks

When this project was initiated, we understood little about the relationship between feeding and fat storage in *C. elegans*. In addition, we had little information about how perception of food is coordinated with internal metabolic needs. Through these studies we now understand that, in *C. elegans*, signals of food availability such as the *daf-7*/TGF- β ligand regulate feeding behavior and metabolism, allowing the animal to adapt to changing environmental conditions. While both feeding behavior and fat storage are regulated by the perception of food availability, these two phenotypes can be dissociated molecularly. This implies that alterations in feeding behavior do not cause changes in fat storage. This research also highlights the ability of animals to anticipate changing environmental conditions, altering metabolism and behavior accordingly. The dissection of the neural and genetic pathways underlying the effects of the TGF- β pathway on fat storage, feeding behavior, and development can act as a paradigm for understanding the relationship between metabolism and behavior in *C. elegans* and possibly other organisms.

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Appendix I: Methods and Protocols

Experimental Procedures

C. elegans Maintenance and Strain Constructions

Nematodes were grown and maintained on NGM plates using OP50 *E. coli* bacteria as food source as described (Brenner 1974). Dauer constitutive strains were maintained at the permissive temperature of 20°C. Although *daf-1(m40)* and *daf-7(e1372)* dauer phenotypes are temperature sensitive, neither the pumping rate nor the fat phenotypes depended on temperature.

Strains were obtained from the CGC (www.cbs.umn.edu/CGC/), OMRF Knockout Consortium (www.mutantfactory.ouhsc.edu/), and the National Bio-resources Project (shigen.lab.nig.ac.jp/c.elegans/). Double mutants of *daf-1(m40)* or *daf-7(e1372)* with each of *tbh-1(ok1196)*, *tdc-1(ok914)*, *tyra-2(tm1846)*, *ser-2(pk1357)*, *ser-3(ad1774)*, *egl-3(gk273)*, *cat-2(e1112)*, *eat-4(ky5)*, *unc-17*, *srw-100(tm2540)*, *srw-100(tm2545)*, B0310.1(tm2480), *prk-2(pk278)*, *srd-45(2197)*, *flp-18(tm2179)* or *cnt-1(tm2313)* were constructed by mating heterozygous males for each genotype to *daf-1(m40)* or *daf-7(e1372)* hermaphrodites, and picking F₂ animals that phenotypically resembled *daf-1(m40)* or *daf-7(e1372)* (dark intestine, egg-laying defective, and constitutive dauer formation). These animals were then screened for molecular mutations in genes of interest by PCR and sequencing. A similar strategy was used for generation of triple mutants starting with combination of double and single mutants. Similarly, *daf-1(m40); daf-12(m20)* mutants were constructed by picking F₂ animals that were egg-laying defective and had a dark intestine but failed to enter dauer state rising from mating of *daf-12(m20)* males and *daf-1(m40)* hermaphrodites. Triple mutants for *daf-1(m40); daf-*

l2(m20); tdc-1(ok941) and *daf-1(m40); daf-12(m20); tbh-1(ok119)* were picked as above and then screened for *tdc-1(ok941)* or *tbh-1(ok119)* deletions by PCR.

Sudan Black B Fat Staining and Quantitation

Animals for sudan black staining were plated on NGM plates as synchronized L1's then raised to young L4 stage for 2 days at 20°C. All Sudan Black B assays were performed at room temperature (22°C) as previously described (Kimura, Tissenbaum et al. 1997) with the following modification: to minimize staining variability, animals from one genotype were labeled with FITC and then fixed and stained in the same tube as unlabeled animals from another genotype. For transgenic lines, fat phenotype was assayed by comparing transgenic animals marked with *Podr-1::dsRED* and non-transgenic animals which were fixed and stained in the same tube. Sudan Black images were collected on a Zeiss Axioplan 2 microscope fitted with a Hamamatsu Orca-AG camera. Staining intensities were quantitated using Improvision Openlab software. Mean pixel intensity was calculated for staining in the region from the first intestinal cells adjacent to the pharynx midway through the animal by the vulva. Background was determined based on pixel intensity of nonspecific staining in the pharynx. Values are reported as mean pixel intensity minus background for about ten randomly selected test and control animals fixed and stained in the same tube.

Pharyngeal Pumping Assay

Screen of RNAi clones: Each of ~400 fat regulatory RNAi clones was screened for pharyngeal pumping defects once by plating synchronized L1 stage larvae on either RNAi or vector control (L4000) bacteria (HT115). Pharyngeal pumping rates of 8

young adult animals was assayed for 10 seconds. ~70 RNAi clones which showed a pharyngeal pumping phenotype in the screen were assayed five additional times to determine if phenotype was reproducible. 30 RNAi clones produced reproducible changes in pharyngeal pumping when RNAied in well-fed N2 animals.

Epistasis with daf-7(e1372), daf-1(m40), eat-4(ad819), and Serotonin. Synchronized L1 wild type, *daf-7(e1372)*, and *daf-1(m40)* animals were placed on either vector control or RNAi that increased feeding rate. Reported values are the average of 32 animals assayed for 10-second intervals from 4 independent experiments. 5mM Serotonin (Sigma) was added onto 6cm RNAi plates with either vector control or test RNAi bacteria. Reported values are the average of 24 animals assayed for 10-second intervals from 3 independent experiments. Epistasis with *eat-4(ad819)* was performed as described for *daf-7(e1372)*. Reported values indicate average of 40 animals assayed for 10 second intervals from 5 independent experiments.

Well-fed condition: synchronized L1 stage larvae were grown on OP50 *E. coli* at low density at 20°C. For experiments using *daf-1* or *daf-7* pharyngeal pumping rates were assayed in well-fed, gravid adults using a Zeiss M² BIO microscope and an electronic counter (Diffcount) to keep track of the number of pharyngeal pumps for individual animals. Animals were grown to adulthood at 20°C then moved to room temperature for pharyngeal pumping assays. Young adult (not yet gravid) animals were assayed in RNAi experiments and animals were grown at room temperature. Pharyngeal pumping of individual animals were counted for 15-second intervals. Normally, pumping rates of ~8-

20 animals were counted per genotype per day. These counts were repeated on at least 3 independent assays performed on separate days.

Food deprived condition: well-fed young animals were washed off plates and rinsed twice with S-basal buffer (Brenner 1974) and maintained in S-basal in a rotating 15ml conical tube for 30 minutes. Animals were then spun into a pellet, rinsed, and plated on unseeded plates. Pumping rates were measured for ~8 animals per genotype 30 minutes post plating. These counts were repeated at least 3 independent times. Reported values are the average of all experiments.

Dauer pheromone treatment: 30 animals grown to the desired stages (late larval stage/young adult) under well fed conditions were transferred to pheromone plates containing 30 ml/ml of a dauer pheromone prep (Golden and Riddle 1982) and 30 ml of OP50 *E. coli* food. These conditions caused dauer entry in ~75% of early larval stage animals. The reported values are averages of 8 animals per genotype per assay. Assays were repeated three independent times. Reported values are the average of all experiments.

Dauer and Egg-retention Assays

Synchronized L1 animals were seeded at low density on plates with plentiful OP50 *E. coli* at 25°C. The plates were maintained at 25°C and the ratio of adults to dauer larvae were counted three days after plating. Dauers were identified based on their visual characteristics (example in Chapter III, Figure 2). To confirm this visual inspection, in

select cases, animals were washed off plates and subjected to 1% SDS treatment and surviving animals were counted. This treatment kills non-dauer animals (Cassada and Russell 1975). To assess egg retention phenotype, animals were synchronized and grown under well-fed conditions at low density. Egg retention was scored in 2 days after animals reached adulthood.

Plasmid and GFP-Reporter Fusion Constructs

Plasmids for tissue reconstitution rescue lines were constructed using Gateway Technology (www.invitrogen.com). The *daf-1* and *ser-2* ORFs were obtained from the *C. elegans* ORFeome (www.openbiosystems.org). The *daf-3* genomic was cloned using *C. elegans* ORFeome primers and the pDONR-221 vector (Deplancke, Dupuy et al. 2004; Dupuy, Li et al. 2004). Promoters for *egl-3*, *bbs-1*, *osm-6*, *glr-8*, *B0280.7*, *glr-1*, *glr-5*, *unc-17*, *glr-2*, *glr-4*, *unc-47*, *ggr-2*, *flp-1*, *dop-1*, *nmr-2*, *myo-2* and *flp-18* were constructed using Promoterome primers and the pDONR-P4-P1R vector (Deplancke, Dupuy et al. 2004; Dupuy, Li et al. 2004). These promoters generally contained 2 kb of genomic sequence upstream of the corresponding gene's ATG start site. Promoters for *daf-1*, *daf-7*, *glr-7*, *tbh-1*, *tdc-1*, and *hsp16.2* were generated using primers to amplify published promoter lengths (Ren, Lim et al. 1996; Gunther, Georgi et al. 2000; Brockie, Madsen et al. 2001; Alkema, Hunter-Ensor et al. 2005).

Rescue constructs were generated using the pDEST-MB14 (*daf-1* and *daf-1;daf-3* rescue) or modified plasmid pKA453 (*daf-1;ser-2* rescue) which lacks the *unc-119* rescue region and contains co-translational GFP fusion. Using the pDEST-MB14 plasmid to obtain *promoter::daf-1-ORF::gfp* translational fusions (Vaglio, Lamesch et al. 2003)

such that *gfp* was fused in frame to the C-terminal end of *daf-1* ORF where the *daf-1* stop codon had been eliminated. Expression patterns of these translational full length fusions were compared to expression patterns of *promoter::gfp* transcriptional fusions constructed by using pDEST-DD04 vector.

Expression patterns of pumping regulatory genes were determined using translational and transcriptional reporter fusions. For translational fusions, primers were used to PCR-amplify genomic regions corresponding to presumptive promoter and the full length gene which was then fused in frame to a GFP reporter followed by an *unc-54* 3'-UTR (Cassata, Kagoshima et al. 1998; Miller, Desai et al. 1999). To capture the presumptive promoter elements for each gene, 1-4kb of genomic region 5' to the start site ATG were examined. For transcriptional fusions, these 5' genomic regions were fused directly to the GFP-*unc-54* 3' UTR.

Cell Ablation

We received cDNA for ICE in a plasmid from the Maricq lab. This was cloned into the pDONR-221 vector with the addition of a stop codon. *ptdc-1::ICE* was constructed using the pDEST-MB14 gateway destination vector. *daf-1(m40)* animals were injected with both *ptdc-1::ICE* and *ptdc-1::gfp*. We obtained several transgenic lines where there was not GFP expression in RIM or RIC however GFP was observed in the UV1 cells indicating successful ablation of RIM and RIC. We also injected N2 with both *ptdc-1::ICE* and *ptdc-1::gfp* however we never obtained successful ablation. *daf-1(m40)* [*ptdc-1::ICE* ; *ptdc-1::gfp*] animals were mated to N2 to obtain ablation of RIM and RIC in N2.

Nile Red Assay

Nile red assays were performed as previously described (Ashrafi, Chang et al. 2003; McKay, McKay et al. 2003; Mak, Nelson et al. 2006). Synchronized L1 larval stage animals were plated on 6cm NGM plates with OP50 bacteria and 1ng/mL Nile red (Molecular Probes). Intestinal Nile red staining was assayed in adults (48-72 hours after plating L1's).

Exogenous Addition of Tyramine and Octopamine

1M stock of Tyramine and Octopamine (Sigma) were diluted to desired concentration (4mM or 2.5mM) by adding 500µL diluted solution to 6cm plates (10mL volume).

Transgenic Animals

For tissue specific rescue experiments, *daf-1(m40)*, *daf-1(m40); daf-3(mgDf90)*, or *daf-1(m40); ser-2(pk1357)* animals were injected with PCR purified plasmid of interest at a concentration of 20ng/ul and co-injection marker *odr-1::dsRED* at a concentration of 30ng/ul. At least three independent lines were generated for each transgene.

Pmgl-1::mCherry and *Pmgl-3::gfp* reporter lines were established by injecting 20ng/ul of each reporter.

To obtain transgenic lines for each of the pumping regulatory gene-GFP reporter fusions, N2 animals were injected with gel purified PCR products at a concentration of 30ng/ul with *rol-6* co-injection marker also at a concentration of 30ng/ul for fusions with

ZK39.7, T05C12.1, *nkat-1*, T21C9.11, Y49A3A.1, K02E7.11, F56H11.6, *dur-1*, and *srw-100*. N2 animals were injected with gel purified PCR products at 30ng/ul and *unc-122::gfp* which expresses in coelomocytes and neurons (URX and occasionally pharyngeal neurons 13 and 15) for fusions with F13B6.1, B0310.1, C09G12.5, and *gpd-3*. Plasmids containing ~2kb of *prk-2* and *cnt-1* promoters were obtained from the Vidal lab and injected with 30ng/ul *rol-6* co-injection marker. In each case, about three independent lines were generated.

Heat Shock Activation of *hsp16.2::daf-1* Transgene

Synchronized L1 stage control and test animals were grown to L3/L4 or the adult stage at 20°C, then plates were moved to a 34°C incubator for 45 minute heat shock. Heat shocked animals were allowed to recover at 20°C for 1 hr. Fat, feeding, and egg retention phenotypes were assayed 30 minutes to 24 hrs post recovery.

Genetic Screening and Molecular Mapping

~1500 *daf-1(m40)* animals were EMS mutagenized (Brenner 1974). ~24,000 resulting F₁ progeny were grown at 20°C and equally divided into 24 pools. These pools were allowed to self fertilize giving rise to ~125,000 F₂ progeny. These animals were visually inspected for dark intestine, egg retention, and Nile red staining at 20°C. Hermaphrodites with the desired phenotypes were singled onto plates and allowed to reproduce. F₁ progeny of these hermaphrodites were then re-examined for the desired phenotypes and F₂ progeny were divided into two groups, one of which was examined for

larval constitutive dauer entry at 26.5°C and the other was assayed for Sudan Black B staining.

The *ft7*, *ft9*, and *ft10* suppressors were crossed into *daf-7(e1372)* animals and backcrossed 2-4 times. For mapping purposes, we generated a CB4856 strain carrying the Bristol *daf-7(e1372)* locus. The *ft7*, *ft9*, and *ft10* suppressors were mapped to chromosome using the DraI mapping technique (Davis, Hammarlund et al. 2005). Subsequent fine mapping used standard single nucleotide polymorphism techniques (Jakubowski and Kornfeld 1999).

Biochemical Assessment of Fat Synthesis

Synchronized *C. elegans* were grown on a mixture of unlabeled bacteria and ¹³C labeled bacteria. Total lipid was purified, converted to fatty acid methyl esters and analyzed by GC/MS (Watts and Browse 2002). The fraction of palmitic, stearic, and vaccenic fatty acid produced via de novo fatty acid synthesis, as opposed to fatty acid obtained from direct dietary absorption, was calculated based on the relative isotope distribution (*Perez and Van Gilst, in preparation*).

Anatomical Methods

Identities of cells expressing gene-reporter fusions were determined by standard anatomical methods including dye filling of amphid neurons to help orient positions of various neurons.

Sudan Black Staining

Grow Worms

- Start with 2-3 6cm plates full of worms (total of 1500 worms).
- Stain L4 animals because the dye looks better when you don't have the eggs staining, in addition the cuticle might be better for fixation. The staining in L4's looks more consistent across the population, which makes comparisons between genotypes easier.
- To further reduce the problems of variability in fixation, fix and stain test and control animals in the same tube by labeling one with FITC. Label worms with FITC putting worms on FITC plates for half an hour (see below for making FITC plates or wormaltas.org).
- Wash worms off the plate in either M9 or S-basal buffer and wash away bacteria (1 or 2 washes)

Fixation

- Wash worms off the plate in either M9 or S-basal buffer and wash away bacteria (1 or 2 washes)
- After worms have washed clean of bacteria- transfer to microfuge tube in 1mL of liquid (M9 or S-basal)
- Incubate worms on ice for ~10 min to stop pumping
- Add 50µl of fresh 10% Paraformaldehyde and freeze immediately in dry ice/ethanol bath (you can store worms at -80 at this point but I've never tried it)
- Freeze thaw worms 3X (freeze in a dry ice/ethanol bath and thaw by placing the tubes in a beaker of warm water). After the final thaw let the animals sit on ice

for 10-20 min or until completely unfrozen. (It is really, really important not to thaw the tubes all the way during any freeze thaw steps- “thaw” should be less than 1 min and only the bottom 100µl of liquid should melt)

Staining

- Wash fixed worms in cold PBS 3-4 times
- Aspirate off PBS and add 1mL 25% Ethanol for 2min, spin, aspirate off 25% EtOH and add 1mL 50% EtOH for 2min, aspirate off 50% EtOH and add 300µl 70% EtOH.
- Add 250ul of saturated sudan black (in 70% ethanol)
- Stain animals overnight
- Spin worms down and aspirate sudan black leaving ~100ul
- Wash once in 70% EtOH to get rid of any sudan black clumps which will not be good for taking pics.
- Mount worms on 2% agarose pad (use a cut 20µl pipette tip to move worms)

Solutions

- Sudan Black ~16mg/ml in 70% EtOH will give a saturated solution. This needs to be filtered (.2um filter) to remove undissolved sudan black.
- 10% Paraformaldehyde: 1g/10ml hot ddH₂O. Add 1-2 drops of 2N NaOH and incubate for 15min at 55 °C. PFA will keep for about a week at 4 °C.
- FITC stock: 20mg/mL solution in dimethylformamide. Take 50ul of FITC stock and dilute in 200ul PBS plate 250ul mix on 6cm seeded plate and let dry. FITC can be purchased from Invitrogen or Fluka as Flourescein isothiocyanate.

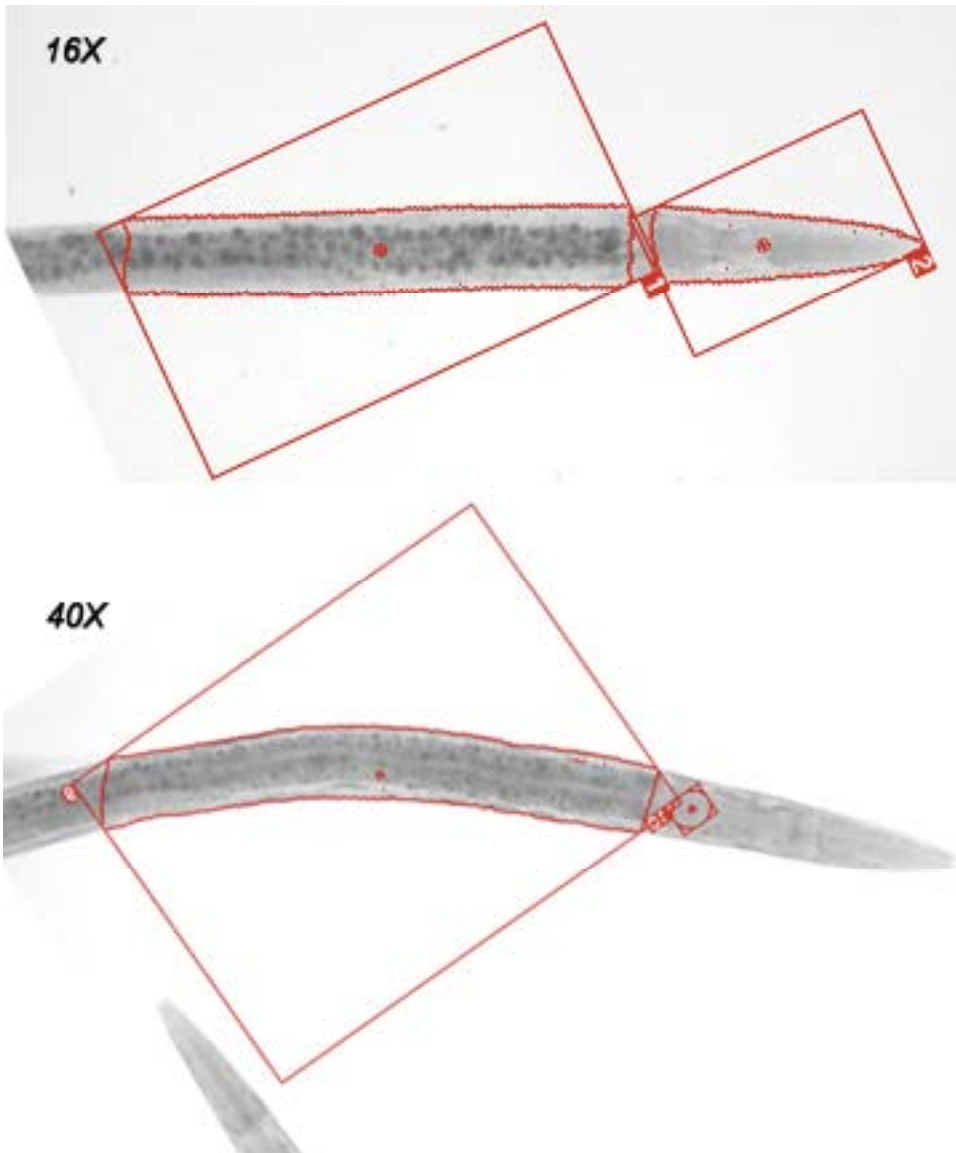
Quantitation of Sudan Black Images

- Fix and stain test and control strains in the same tube by labeling 1 genotype with FITC as described in staining protocol.
- Image test and control animals using the same microscope settings. Since Sudan Black staining can vary between fixations you may want to change your microscope settings between experiments however animals to be compared by quantitation must be imaged in the same manner
- On average try to obtain measurements from 10 animals per genotype
- Once you have acquired your images you can calculate the sudan black intensity in Openlab by determining the average (mean) pixel intensity over a given area.
 - Select area from the start of the intestine to the vulva using the lasso tool (in the top left corner of Openlab window)
 - Once the area is selected open the measurements window by going to the Image toolbar select show measurements. At this point the measurements window will appear. In the measurements window select to ignore white pixels and ignore black pixels. (You will only need to do this selection once when measuring many images.) In order to measure your selected area click the little camera icon in the measurements window.
 - The background staining can be determined by the intensity of staining in the pharynx. This measurement can be obtained as described above. For images taken at 16X use the lasso tool to select the entire pharynx region.

For 40X images use the circle tool to select a portion of the posterior pharynx (examples of intestine and pharynx selection are shown below)

- For each animal obtain intestine and pharynx measurements.
- Once you have acquired the measurements move the data to excel to evaluate further
 - Subtract the mean pixel intensity of the pharynx measurement from the mean pixel intensity of the intestine.
 - Average the intestine - pharynx values for 10 test and 10 control animals then determine the ratio of test intensity to control intensity to obtain a value that is Relative Sudan Black Staining (% of Control).
 - Remember since more sudan black (darker stain) means more fat low pixel intensity will correspond to more sudan black staining.
- For every strain assayed you must compare test and control that have been fixed and stained in the same tube. Unfortunately, since sudan black staining can be variable across experiments it is inappropriate to obtain measurements from control and compare that to several test genotypes fixed in separate tubes.

Area Selection for 16X and 40X images



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Appendix II: *C. elegans* strains

Strains Used in study of TGF- β regulation of feeding behavior and fat storage

Strain	Fat Content ^a	Pumping Rate (% WT)	Egg laying phenotype ^b	Reference
<i>daf-7(e1372)</i>	increased	81 $\pm$ 2.9	+++	Riddle et al., 1981
<i>daf-1(m40)</i>	increased	78 $\pm$ 3.4	+++	Riddle et al., 1981
<i>daf-4(m63)</i>	increased	87 $\pm$ 2.9	+++	Golden and Riddle, 1984
<i>sma-6(wk7)</i>	wild type	100 $\pm$ 3.2	+	Krishna et al., 1999
<i>daf-12(m20)</i>	wild type	99 $\pm$ 2.6	+	Riddle et al., 1981
<i>daf-3(mgDf90)</i>	wild type	98 $\pm$ 2.9	+	Patterson et al., 1997
<i>tbh1(ok1196)</i>	wild type	97 $\pm$ 2.8	+	OMRF ^c
<i>tdc-1(ok914)</i>	wild type	95 $\pm$ 3.6	+	OMRF ^c
<i>tyra-2(tm1846)</i>	wild type	100 $\pm$ 2.8	+	NBP ^d
<i>ser-2(pk1357)</i>	wild type	99 $\pm$ 4.3	+	Tsalik et al. 2003
<i>goa-1(n363)</i>	wild type	75.4 $\pm$ 5.9	egg laying	Segalat et al.,

			constitutive	1995
<i>dgk-1(sy428)</i>	wild type	83 ± 3.6	egg laying constitutive	Hajdu- Cronin et al., 1999
<i>eat-4(ky5)</i>	wild type	72 ± 7.4	+	Lee et al., 1999
<i>mgl-1(tm1811)</i>	wild type	98 ± 5.5	+	NBP ^d
<i>mgl-2(tm0355)</i>	wild type	90 ± 7.1	+	NBP ^d
<i>mgl-3(tm1766)</i>	wild type	101 ± 2.8	+	NBP ^d
<i>daf-7(e1372); daf-12(m20)</i>	increased	83 ± 4.2	+++	Riddle et al., 1981
<i>daf-7(e1372); daf-3(mgDf90)</i>	wild type	98 ± 3.1	+	this study
<i>daf-1(m40); daf-3(mgDf90)</i>	wild type	99 ± 2.8	+	this study
<i>daf-1(m40); daf-12(m20)</i>	increased	80 ± 3.4	+++	this study
<i>daf-7(e1372); tbh-1(ok1196)</i>	increased	94 ± 3.4	+++	this study
<i>tdc-1(ok914); daf-7(e1372)</i>	increased	95 ± 3.6	+++	this study
<i>daf-1(m40); tbh-1(ok1196)</i>	increased	89 ± 2.8	+++	this study
<i>tdc-1(ok914); daf-1(m40)</i>	increased	91 ± 3.0	+++	this study
<i>tdc-1(ok914); daf-7(e1372); daf-12(m20)</i>	increased	95 ± 3.7	+++	this study
<i>daf-1(m40); daf-12(m20); tbh-1(ok1196)</i>	increased	91 ± 4.2	+++	this study

<i>tdc-1(ok914); daf-1(m40); daf-12(m20)</i>	increased	92 ± 3.1	+++	this study
<i>daf-1(m40); tyra-2(tm1846)</i>	increased	74 ± 3.9	+++	this study
<i>daf-1(m40); ser-2(pk1357)</i>	increased	88 ± 2.9	+++	this study
<i>daf-1(m40); ser-2(pk1357); tyra-2(tm1846)</i>	increased	87 ± 3.0	+++	this study
<i>egl-30(ft9); daf-7(e1372)</i>	wild type	78 ± 5.8	egg laying constitutive	this study
<i>daf-7(e1372); dgk-1(sy428)</i>	wild type	78 ± 7.4	egg laying constitutive	this study
<i>goa-1(n363); daf-7(e1372)</i>	wild type	70 ± 3.7	egg laying constitutive	this study
<i>daf-7(e1372); eat-4(ky5)</i>	reduced relative to <i>daf-7(-)</i>	66 ± 3.5	+++	this study
<i>daf-7(e1372); mgl-1(tm1811)</i>	reduced relative to <i>daf-7(-)</i>	77 ± 3.7	+++	this study
<i>mgl-2(tm0355); daf-7(e1372)</i>	increased	80 ± 3.9	+++	this study
<i>daf-7(e1372); mgl-3(tm1766)</i>	reduced relative to <i>daf-7(-)</i>	79 ± 4.5	+++	this study
<i>daf-7(e1372); mgl-3(tm1766);</i>	reduced	77 ± 4.8	+++	this study

<i>mgl-1(tm1811)</i>	relative to <i>daf-7(-)</i>			
<i>daf-7(e1372); cat-2(e1112)</i>	increased	N/D	+++	this study
<i>daf-7(e1372); egl-3(gk238)</i>	increased	N/D	+++	this study

^aFat levels were determined by Sudan Black B. staining. Comparisons are made relative to wild type (N2) animals except when noted relative to *daf-7(e1372)*. Strains denoted as having an “increased” phenotype retained ~2.5 fold more fat than wild type strains. Quantitative measures for fat contents of strains denoted as “reduced relative to *daf-7(-)*” are shown in Figure 5D.

^b Retention of eggs relative to wild type. Young, well-fed wild type animals retained ~10 eggs on average. This phenotype is denoted as (+). Mutants such as *daf-1(m40)* retained ~24 eggs. Excess egg retention phenotypes of such mutants are designated as (+++). Egg laying constitutive animals displayed an empty uterus phenotype.

^cOMRF: Oklahoma Medical Research Foundation, The *C. elegans* Gene Knock-out Consortium (<http://celeganskoconsortium.omrf.org/>).

^dNBP: National Bioresources Project (<http://www.grs.nig.ac.jp/c.elegans/index.jsp>).

Additional Strains obtained or created

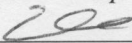
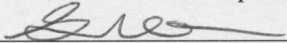
Strain	Pharyngeal Pumping Rate (% N2)	Fat Phenotype (method used to assay phenotype)
<i>cnt-1(tm2313)</i>	99 ± 5.2	none (nile red)
<i>srd-45(tm2196)</i>	102 ± 4.4	increased fat (nile red)
<i>tph-1(mg280)</i>	71.8 ± 8.6	
<i>tph-1(mg280); cnt-1(tm2313)</i>	74 ± 5.5	
<i>tph-1(mg280); srd-45(tm2196)</i>	69.2 ± 8.6	
<i>prk-2(pk278)</i>	106 ± 3.6	none (nile red)
<i>daf-1(m40)</i>	71 ± 6.3	increased fat (sudan black)
<i>daf-1(m40); prk-2(pk278)</i>	86 ± 4.7	increased fat (sudan black)
<i>daf-1(m40); flp-18(tm2179)</i>	68 ± 5.8	increased fat (sudan black)
<i>daf-7(e1372)</i>	79.1 ± 3.7	increased fat (sudan black)
<i>daf-7(e1372); cnt-1(tm2313)</i>	82.3 ± 8.6	
<i>daf-7(e1372); srd-45(tm2196)</i>		
<i>tax-6(p675)</i>	68 ± 7.4	
<i>dur-1(ok1010)</i>	70 ± 12	
<i>srw-100(tm2540)</i>	77 ± 10	increased fat (nile red)

<i>srw-100(tm2545)</i>	91 ± 3	increased fat (nile red)
B0310.1(<i>tm2480</i>)	77 ± 4.2	increased fat (nile red)
B0310.1(<i>tm2480</i>); <i>daf-7(e1372)</i>		increased fat (sudan black)
<i>srw-100(tm2540)</i> ; <i>daf-7(e1372)</i>		increased fat (sudan black)
<i>srw-100(tm2545)</i> ; <i>daf-7(e1372)</i>		increased fat (sudan black)
B0310.1(<i>tm2480</i>); <i>tdc-1(ok914)</i>		
<i>srw-100(tm2540)</i> ; <i>daf-3(mgDf90)</i>		
B0310.1(<i>tm2480</i>); <i>daf-7::gfp</i>		

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