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Endogenous RNAi pathways are required in neurons for dauer formation in Caenorhabditis elegans

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Abstract

Animals can adapt to unfavorable environments through changes in physiology and behavior. For *Caenorhabditis elegans* nematodes, environmental conditions perceived early in development determine whether the animal enters the reproductive cycle or enters into an alternative diapause stage called dauer. Here we demonstrate that endogenous RNA interference (endo-RNAi) pathways play a pivotal role early in the life cycle of worms in the detection of environmental conditions and mediating the appropriate developmental decisions. Our findings illustrate that functional endo-RNAi pathways contribute to the regulation of dauer formation in larvae when exposed to stress and recovery from the dauer stage when the conditions improve. Our experiments demonstrate that disruption of the *Mutator* proteins or the nuclear argonaute CSR-1 result in differential dauer-deficient phenotypes that are dependent upon the environmental stress. We found that these RNAi components function in individual neurons upstream of TGF-β and insulin signal transduction pathways to positively regulate G protein genes that are required to make dauer formation decision, suggesting a role for the distributed neuronal circuit in regulating this decision. Our results also suggest that endo-RNAi governs dauer recovery decisions in pheromone induced dauers when conditions are favorable. Together, our data suggest a model wherein the CSR-1 pathway promotes expression of genes required for the detection and signaling of environmental conditions, and highlight a mechanism whereby RNAi pathways mediate the link between environmental stress and adaptive phenotypic plasticity in animals.

Endogenous RNAi Pathways Are Required in Neurons For Dauer Formation in *Caenorhabditis elegans*

by

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Chapter 1

Background

1.1 Polyphenism is expressed in adverse environments

Animals are capable of undergoing physiological and behavioral changes to adapt to adverse environments, a process known as allostasis (Sterling and Eyer,1988). One such adaptation is the expression of polyphenism, or alternative developmental morphs, in a population of genetically identical organisms (Michener 1961, Mayr 1963). There are excellent examples reflecting the effects of environment on phenotypic plasticity leading into differential adult phenotypes. Caste determination in social insects such as honeybees is one such example of polyphenic development (Nijhout 1998, Nijhout 1999, Amdam *et al*. 2004). Female honeybees can either be designated to be worker bees or the single dominating queen bee. Worker bees carry out all the tasks to sustain a population, including collection of food and caring for the larvae. On the other hand, the queen bee mates with various potent males to generate genetic diversity and lays eggs (Oster and Wilson 1978). Although genetically identical, the polyphenism state of adult honeybees is dependent on the type of diet and the amount of moisture in the food that they received as larvae (Dietz *et al*. 1971, Hrassnigg *et al*. 2005, Maleszka 2014). The supply of the superior 'royal jelly' in the diet allows the larvae to develop into a queen; whereas the remaining larvae, which receive more of the p-coumaric acid are destined to become worker bees (Chittka and Chittka 2010, Kamakura 2011, Mao *et al*. 2015). The disparity in development between the classes is speculated to be due to the

differences in food sensing pathways prevailing in larval stages that are later on translated into phenotypic differences in adulthood (Wheeler *et al*. 2006, Patel *et al*. 2007, de Azevedo and Hartfelder 2008, Wolschin *et al*. 2011).

A second example of expression of polyphenism is seen in *Daphnia longispina,* which are water fleas that can exist in two forms: with helmet and long spines (armored), or without helmet and short spines (unarmored), depending on the presence of specific chemicals they might have encountered at the time of molting from juvenile stage. *Daphnia* possess antennules that detect the chemicals released by predators such as the aquatic insect *Notonecta glauca*, which activates secretion of neurotransmitter dopamine and the development of the appendages. The development of the defense mechanism is speculated to interfere with the predators' feeding mechanism (Juday 1910, Brewer *et al.* 1999 and reviewed in Ranta *et al*. 1993, Ebert 2005, and Weiss *et al*. 2012).

Phenotypic plasticity is also demonstrated by the seasonal differences in the wing patterns of adult *Pieris* butterflies (Shapiro 1976, Nijhout 1999). *Pieris* can exhibit variable wing melanin patterns determined by the temperature and/ or photoperiod that they were exposed to as larvae (Weismann 1882, Goldschmidt 1938, Shapiro 1976). This effect is believed to be important in adults for thermoregulatory adaptation to the cold and warm temperatures (Kingsolver and Wiernasz 1991), and increasing the fitness of the butterflies (Stearns 1989). These examples of polyphenisms illustrate how animals can adapt their physical characteristics and developmental trajectory in response to unfavorable environmental conditions; however the molecular mechanisms regulating these phenomena is still under investigation.

Given that polyphenisms can occur in isogenic populations of animals, epigenetic mechanisms, such as RNA interference (RNAi) and DNA methylation, are hypothesized to regulate the expression of alternative phenotypic morphs in response to environmental conditions (West-Eberhard 2003, Wang *et al.* 2006, Kronforst *et al.* 2008, Kucharski *et al.* 2008, Moczek and Snell-Rood 2008, Bonasio 2012, Humann *et al.* 2013).

For instance, female honeybee larvae that received royal jelly diet exhibit distinct DNA methylation signatures than those which receive an ordinary diet ultimately leading to different developmental trajectories (Kucharski *et al*. 2008). In pea aphids, unwinged and winged morphs develop in response to favorable or unfavorable environments, respectively (Reviewed in Müller 2001, Brisson 2010). Although genetically identical, winged and unwinged female populations exhibit differential DNA methylation patterns and expression of genes that are associated with regulation of juvenile hormone (JH), which has been implicated in wing polyphenism (Walsh *et al.* 2010). In addition, polyphenic transitions of locusts from solitary phase to gregarious (swarm formation) depends upon the differential accumulation of small RNAs in the two phases (Wei *et al.* 2009). Despite these examples, the molecular mechanisms that govern gene targeting and regulation by epigenetic pathways in response to environmental conditions are not well understood.

1.2 Dauer formation in *C. elegans* is an example of polyphenism

Caenorhabditis elegans is an excellent model system to study the molecular mechanisms regulating polyphenism, as we can take advantage of their alternative developmental trajectories, which are determined by environmental conditions after

hatching. In favorable growth conditions, worms proceed continuously through four larval stages (L1-L2-L3-L4) to form reproductive adults (control adults). In unfavorable conditions, such as low food availability, high temperatures, or high pheromone concentrations, larval L1 worms will proceed to the extended L2d stage. In this stage the larvae prepare to make the developmental decision to either molt to L3 if the conditions improve, or enter an alternative developmental stage called dauer if the

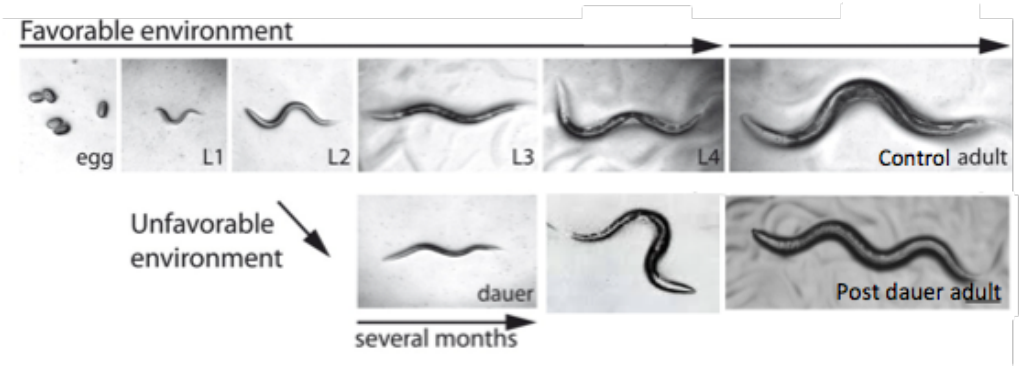


Fig. 1. Effect of early stress in *C. elegans*. Environmental stresses namely, overcrowding (increased pheromone), temperature, and starvation determine the developmental trajectory in *C. elegans*. Figure modified from Fielenbach and Antebi, 2008.

environment continues to be unfavorable (Cassada and Russell 1975; Golden and Riddle 1982; Golden and Riddle 1984a, b, c). When in dauer stage, worms develop a thick cuticle with alae (a longitudinal structure appearing as a protruding ridge) that protects them from desiccation and harsh environmental conditions (Cassada and Russell 1975). Dauers are non-feeding (no pharyngeal pumping), non-aging, and thought to be essential for dispersal in an environment unfavorable for reproduction (Klass and Hirsh 1976; Larsen 1993; Frézal and Félix 2015). Once conditions improve, animals may exit dauer to resume continuous development to L4 followed by growth into adulthood (postdauer adults) (Cassada and Russell 1975; Golden and Riddle 1982; Golden and Riddle 1984a, b, c) (Fig. 1).

Previous genetic screens performed to characterize the molecular pathways leading to dauer formation categorized two types of mutants exhibiting inappropriate dauer formation (*daf*) phenotypes. Strains that form significantly fewer dauer larvae than wild-type in response to environmental stress are considered dauer deficient (*daf-d*), while dauer constituent (*daf-c*) animals can form dauers even in the absence of environmental stress (Riddle *et al.* 1981, Vowels and Thomas 1992). An additional category of mutants in the dauer formation pathways is defective in dauer morphogenesis and categorized as having "partial dauer" phenotypes. Partial dauer larvae exhibit intermediate phenotypes between dauer and non-dauer and may not possess all the salient features of the dauer larvae. For example, partial dauers often complete normal dauer alae formation accompanied by radial constriction of the body, but fail to remodel their pharynx (Cassada and Russell 1975, Antebi *et al*. 2000, Gerisch *et al*. 2001, Jia *et al*. 2002, Ohkura *et al*. 2003, Li *et al*. 2004, Rottiers *et al*. 2006, Tennessen *et al*. 2010). Also, partial dauers exhibit a dearth of fat accumulation and display irregular pharynx pumping (Vowels and Thomas 1992, Ogg *et al*. 1997). Wild-type dauer larvae are resistant to 1% SDS detergent treatment based on two characteristics that dauer larvae possess: a thick cuticle and inability to ingest the detergent due to sealing of the mouth and absence of pharyngeal activity. However, some partial dauers do not escape this treatment when subjected to SDS for extended amounts of time ($\geq$ 40 minutes) due to their failure to complete dauer morphogenesis (Albert and Riddle 1988, Nika *et al*. 2016).

Given the various morphological and behavioral changes brought about in adverse environments, *C. elegans* like other animals have developed strategies for

adaptation and hence are an apt example of polyphenism. Switching of animals from continuous developmental state to dauer state results in modifications at a molecular level and neurosensory level, and is associated with visible developmental and behavioral changes in postdauer adults (Hall *et al*. 2011, Hall *et al*. 2013, Sims *et al*. 2016). The dormancy in dauers helps worms preserve energy, although the neuromuscular stimuli are completely functional, and indicates the full intention for survival and dispersal (Cassada and Russell 1975, Klass and Hirsh 1976, Larsen 1993, Frézal and Félix 2015). Additionally, entry into dauer stage also causes remodeling of the cilium structure of the head neurons to increase sensitivity to environmental stimuli (Albert and Riddle 1983, White *et al*.1986). *C. elegans* developmental trajectory is yet another example of effects of the early life environment on adult behavior and development.

1.3 Head neurons detect environmental signals in *C. elegans*

C. elegans contains 302 neurons, out of which 11 pairs of bilaterally symmetrical chemosensory neurons in the head are referred to as amphid neurons, which possess ciliated dendrites that extend to the nose of the animal (Ward *et al*. 1975, Ware *et al*. 1975, Perkins *et al*. 1986, Bargmann and Mori 1997)(Fig. 2). Laser ablation studies of subsets of amphid neurons in L1 staged worms allowed identification of neurons pertinent to dauer developmental decisions, namely, ASG, ADF, ASI and ASJ (Bargmann and Horvitz 1991). These neurons express components of the insulin-like (Kimura *et al*. 1997) and TGF-β (Ren *et al*. 1996) pathways to trigger an appropriate developmental response based on the environmental conditions (Golden and Riddle 1982, Golden and Riddle 1984a). They can regulate dauer phenotypes by either

repressing or promoting dauer formation. Under favorable conditions of low pheromone concentration, neurons ASG, ADF, and ASI actively repressed dauer formation.

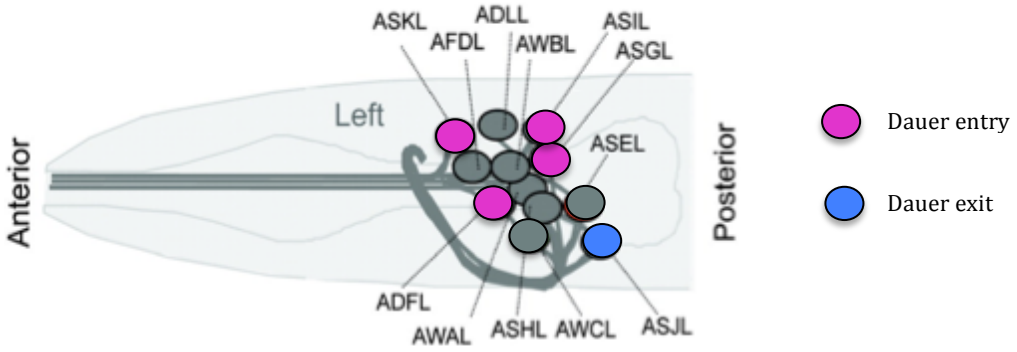


Fig. 2. Requirement of amphid neurons in dauer formation and dauer exit. Amphid neurons in the head of *C. elegans* are centers of receiving environmental cues and initiating dauer formation pathways. Colored neurons in this figure have been implicated in dauer formation and exit (Bargmann et al. 1991). Figure modified from Ortiz *et al*. 2006.

However, when the conditions were unfavorable, these neurons could be de-repressed resulting in entry into dauer state. ASJ on the other hand was found to be dormant in non-inducing conditions and active when nematodes are subjected to high amount of pheromone. This dauer promoting neuron is also required for recovery from dauer when the environmental conditions become favorable (Bargmann and Horvitz, 1991). A subset of the neurons that promote dauer formation have also been implicated in detection of dauer inducing conditions including individual pheromone molecules (ASI and ASK) and starvation stress (ASI) (Clark *et al*. 2006, Kim *et al*. 2009, McGrath *et al*. 2011, Park *et al*. 2012, Jang *et al*. 2012, Neal *et al*. 2015).

1.3.1 Pheromone is the primary inducer of dauer formation

Pheromones are molecules released by animals into the external environment that can bring about both primer and releaser effects. Primer effects are slow, long

lasting changes in physiology and behavior due to pheromone-mediated regulation of genes affecting the endocrine, reproductive and neurological systems. On the other hand, releaser effects of pheromones are fast acting and can elicit an immediate behavioral response (Wilson 1971, Wyatt 2003, Grozinger *et al*. 2003, Alaux *et al*. 2009). An example of releaser effects of pheromones found in nature is the response of male silk moths when exposed to pheromone released by their female counterparts. The presence of a sex attractant in the pheromone caused fluttering of wings and dance like behavior in males. The pheromone responsible for this was soon extracted and became the first pheromone to be isolated from any animal (Sandler *et al*. 2000). Examples of the primer effects can be seen in honeybees. Adult honeybees release a chemical 'forager pheromone', which can delay the food foraging habits in younger bees that can in turn affect the work dynamics in the honeybee population (Leoncini *et al*. 2004).

Effects of pheromone-induced behavior and development in *C. elegans* are also excellent examples of releaser and primer effects. *C. elegans* secretes pheromone throughout its lifetime into the environment that is a blend of hydrophilic ascaroside (ascr) molecules containing 3 to 9 carbon side chains, and the composition of the pheromone differs based on the cultivation environment of the animals (Golden and Riddle 1982, Golden and Riddle, 1984a, Jeong *et al.* 2005, Schroeder 2006, Butcher *et al.* 2007, Butcher *et al.* 2008, Srinivasan *et al*. 2008, Pungaliya *et al.* 2009). Known pheromone molecules are named C3 (ascr #5), C6 (ascr #6), C7 (ascr #1), and C9 (ascr #3) based on the number of carbon atoms in the fatty acid chain attached to the ascaroside sugar (Fig. 3). Pheromone accumulation is interpreted by the nematodes as

a measure of population density (Golden and Riddle, 1982, Golden and Riddle, 1984a). Dauer pheromone is the primary cue for dauer formation, and is an example of primer effects of pheromone. Pheromone molecules have varying effects on dauer formation depending on types and concentration of ascaroside components present (Jeong *et al*. 2005, Butcher *et al*. 2007, Butcher *et al*., 2008), with ascr#5 and ascr#3 being the two main components identified to be required primarily for dauer developmental decisions (Butcher *et al*. 2007, Kim *et al*. 2009).

Releaser effects are also evident in *C. elegans* when observing adult behaviors in response to pheromone. High concentrations of the pheromone component ascr#3

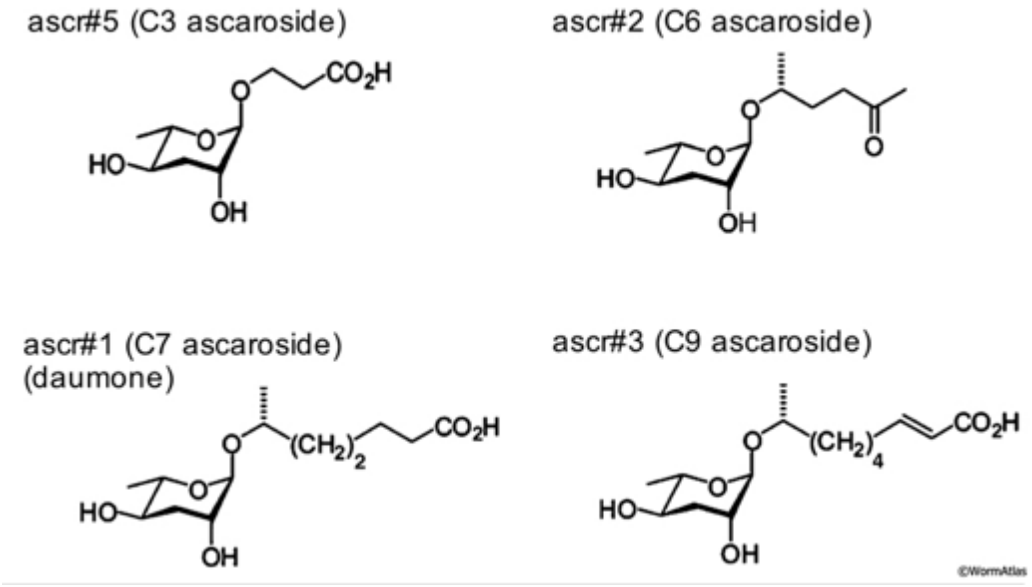


Fig. 3. Individual ascaroside molecules of pheromone identified in *C. elegans*. Pheromone molecules have been shown to elicit dauer formation in larvae and behavioral response in adults. Figure from Wormbook-Von-Reuss *et al*. 2012.

can elicit sex-specific behaviors in adult *C. elegans*. Hermaphrodites avoid high concentrations of ascr#3, while males are attracted to it. The different neuromodulatory

states of the two sexes allow interpretation of the pheromone stimulus differently to generate distinct behavioral responses (Jang *et al*. 2012). Our lab has shown that releaser effects of hermaphrodites are modulated as a result of passage through dauer stage. Postdauer adults exhibit a decreased avoidance behavior in response to ascr#3 compared to continuously developing worms (Sims *et al*. 2016).

Additionally, the different amphid neurons are able to sense individual pheromone molecules. For instance, ASI can detect ascr#5 and ascr#2 molecules and individual ascr#3 component can be detected by ADL (in adults) (Kim *et al*. 2009, Thomas and Robertson, 2008, Park *et al*. 2012, Jang *et al*. 2012). ASK neurons are shown to detect ascr#1, ascr#2, ascr#3, and ascr#5 to some extent for pheromone-induced dauer formation (Kim *et al*. 2009). Pheromone components bind to distinct receptors on the neurons to initiate signal transduction pathways for various developmental and behavioral outcomes. This is consistent with the findings that amphid neurons express G protein coupled receptors (GPCRs) on their surfaces to bind to individual ascaroside molecules to induce dauer formation (Discussed in section 1.4.2).

1.3.2 Temperature is an important physical variable in the *C. elegans* life cycle

C. elegans dauer developmental decision is also dependent on temperature in addition to high pheromone concentrations. Wild-type *C. elegans* dwell in temperatures ranging from 15 to 20°C (Hedgecock and Russell 1975, Ailion and Thomas, 2000). The probability of dauer formation is increased as the cultivation temperature increases from 25°C, substantially enhanced at 27°C, and this outcome can be independent of pheromone concentration (Ailion and Thomas, 2003).

The neural and molecular mechanisms underlying dauer formation due to temperature stress are yet to be fully understood (Golden and Riddle, 1984a, b, Ailion and Thomas, 2000, Ailion and Thomas, 2003). Ablation experiments have demonstrated that removal of the AFD sensory neurons resulted in reduced responsiveness to elevated temperature causing cryophilic phenotypes (Chung *et al*. 2006). AFD neurons are required by worms to distinguish temperature differences of as little as 0.05°C (Ramot *et al*. 2008, Kimura *et al*. 2004, Clark *et al*. 2006). The complex brush like cilium of the AFD sensory endings may act as a sensor to detect temperature changes. Specific thermosensory receptors in cilial ends of thermosensory neurons are yet to be explored although the search is ongoing (Coburn and Bargmann 1996, Komatsu *et al*. 1996, Clark *et al*. 2006, Inada *et al*. 2006). However, few studies have found that mutations in guanynyl cyclases *gcy-8*, *gcy-18*, and *gcy-23* required in cGMP signaling in AFD signal transduction failed to exhibit temperature induced currents and hence defective thermosensation behavior (Hedgecock and Russell 1975, Mori *et al*. 1995, Kimura *et al*. 2004, Ramot *et al*. 2008, Wasserman *et al*. 2011). More studies have emerged implying the role of AWC neurons in modulating AFD neurons in temperature dependent behaviors (Biron *et al*. 2008). However, how the underlying molecular mechanisms in thermosensation modulate dauer formation decisions is not yet understood.

1.3.3 Worms can survive starvation by opting to enter diapause

Availability of nutrients can determine critical life history choices an animal makes. The adaptive nature of *C. elegans* life cycle allows the worms to enter diapause at several stages of their lifetime when experiencing low food availability. Animals can

arrest in L1 stage when they hatch in the absence of food (Baugh and Sterberg 2006) and may enter the dauer stage after L2 due to starvation (Johnson *et al*. 1984, Baugh 2013). Dauers resulting from starvation condition look distinct from dauers formed in the presence of food. Starvation induced dauers appear paler compared to pheromone dauers, a phenotype attributed to the accumulation of fat deposits (Hu *et al*. 2007). Worms encountering starvation in L4 stage will proceed to adulthood but are driven into reproductive diapause and cease laying eggs (Félix *et al*. 2010). Food availability information along with pheromone concentration and temperature conditions needs to be integrated into the sensory system to make an appropriate dauer entry decision in early larval stages.

There are limited studies that have tried to understand the molecular mechanisms underlying dauer formation in response to starvation. Studies in Neal *et al.* 2015 identified activity of one of the key molecules, calcium/calmodulin-dependent protein kinase I (CMK-1), which is widely expressed in neurons to transduce 'nutrient availability signals' for dauer formation. In wild-type worms, starvation conditions cause the transient sequestration of CMK-1 to the nucleus in AWC neurons, which in turn affect regulation of expression of ILP genes *ins-26* and *ins-35* in AWC. The ILP signals can carry this 'food information' to downregulate growth promoting *daf-28* ILP gene in the ASJ neuron to ultimately make the dauer formation decision. In parallel, CMK-1 also functions cell autonomously in ASI to regulate *daf-7* gene expression. Moreover, *cmk-1* mutants exhibit downregulated *daf-7* and *daf-28* as seen in starved or dauer promoting conditions (Neal *et al*. 2015).

1.3.4 Food availability is important for dauer recovery

Like entry into dauer stage, exit from dauer stage also requires assessment of environmental cues, *i.e*., pheromone concentration, availability of food, and temperature (Golden and Riddle 1984b, c). While pheromone concentration is the main trigger for dauer formation, the main cue for worms to exit dauer stage is presence of 'food signals' emanating from live *E. coli* (Golden and Riddle 1982). Indeed, pheromone and food signals show antagonistic effects on dauer recovery (Golden and Riddle 1982). Unlike the isolated pheromone molecules, the identity of the 'food signal' is yet to be fully understood. Evidence suggests that fatty acids secreted from the bacteria augment the food signals to encourage dauer recovery (Kaul *et al*. 2014). Similarly, temperature plays a collaborative role with food and pheromone in regulating dauer recovery. For example, when the culture temperature is lowered from 25°C to 15°C in the presence of exogenous pheromone and food, worms will initiate exit from dauer. Increasing the temperature further (above 25°C) however does not trigger dauer recovery (Golden and Riddle 1984b). Wild-type animals become committed to exiting dauer stage within 40-50 minutes after exposure to fresh food source and begin pharynx pumping within 3 hours for the transition to postdauer L4s (Golden and Riddle 1984b).

1.3.5 G protein coupled receptors bind to pheromone to activate downstream pathways

Generally, G protein coupled receptors (GPCR) play a pivotal role in detection of environmental stimuli and mediating sensory functions of light perception, olfaction, and pheromone signaling (Buck and Axel 1991, Jaffé 2012, Dong *et al*. 2001). Receptors in the amphid neurons represent the first interface between environment and the nervous system (Zwaal *et al*. 1997). In *C. elegans* neurons, GPCRs span the plasma membrane

of cilia and serve to detect specific environmental stimuli, including individual pheromone components. About 950 *C. elegans* genes are predicted to encode seven transmembrane chemoreceptors based on the genome data available (Waterston 1998, Troemel *et al*. 1995, Troemel *et al*. 1997). They are grouped as *sra*, *srb*, *srd*, *sre*, *srg*, *sro*, and *str* based on their sequence similarity (Bargmann 1998) where the 'sr' stands for serpentine chemosensory receptor. Out of these there are roughly 400 genes that are predicted to code for GPCRs (Troemel *et al*. 1997). This gives scope for recognition of various environmental cues by the multiple families of receptors that function in selected number of amphid neurons (Buck and Axel 1991). Therefore, a large number of receptors function in individual neurons devoted to identifying diverse environmental stimuli in *C. elegans* unlike in mammals where the one neuron-one receptor concept applies (Mazzoni *et al*. 2005).

When bound to a ligand, the cytoplasmic portion of GPCRs activate their associated G proteins which function to transduce the intracellular sensory signal (Koelle 2016). There are 20 Gα, 2 Gβ, and 2Gγ distinct heterotrimetric G proteins that interact with GPCRs to trigger downstream intracellular effectors. For instance, a current model suggests GPCRs residing in amphid neurons associate with G proteins to activate the guanylyl cyclase (Thomas *et al*. 1993, Birnby *et al*. 2000), TGF-β (Patterson *et al*. 1997) and insulin signaling pathways (Kimura *et al*. 1997) to regulate dauer formation, metabolism, stress resistance, and aging throughout the life cycle. Mutations in G proteins, GPA-1 (G protein α), ODR-3 (G protein α), and GPC-1 (G protein γ), resulted in reduced sensitivity to pheromone as demonstrated by high pheromone dauer formation assays. In contrast, overexpression of *gpa-11* (guanynyl cyclase) results in a

daf-c phenotype. Disruption of some of these proteins (ODR-3 and DAF-11) also showed additional phenotypes like alteration of longevity via DAF-16 FoxO (Lans and Jansen 2007).

GPCRs that are required for dauer formation are expressed in different neurons. SRBC-64 and SRBC-66 were the first GPCRs to be identified for responding to dauer pheromone. Loss of *srbc-64* and closely related gene *srbc-66* led to a strong *daf-d* phenotype in response to ascr#2 and acsr#3 but exhibited wild-type levels of *daf* phenotype in presence of ascr#5. SRBC-64 and SRBC-66 localize to the ciliary ends of ASK to detect ascarosides ascr#2 and ascr#3, and associate with GPA-2 and GPA-3 to activate downstream signaling pathways to mediate dauer formation (Thomas and Robertson 2008, Kim *et al*. 2009,). SRG-36, SRG-37, and DAF-37 are ASI-specific GPCRs thought to detect ascr#2 and ascr#5 pheromone molecules (Kim *et al*. 2009, McGrath *et al*. 2011, Park *et al*. 2012). The exact intracellular mechanisms regulating downstream pathways when pheromone binds to these receptors are yet to be fully understood.

1.4 Signal transduction pathways regulate dauer formation

Dauer formation pathways consist of a complex network of genes that positively or negatively regulate dauer formation. Genetic screens identifying genes required for dauer formation have characterized mutants exhibiting *daf-d* and *daf-c* phenotypes (Albert *et al*. 1981, Riddle *et al*. 1981, Swanson and Riddle, 1981). Furthermore, epistasis studies of mutants with *daf* phenotypes have revealed that activity of four complex networks of endocrine pathways, conserved among animal species, regulate the dauer developmental decision (Reviewed in Hu *et al*. 2007, Fielenbach and Antebi

2008) (Fig. 4). The detection of the environmental stresses results in altered signaling of the following pathways, promoting dauer formation in adverse conditions.

1.4.1 Insulin-like signaling

Insulin-like signaling is an extensively studied signal transduction pathway that is evolutionarily conserved among animals regulating critical metabolic decisions, development, and lifespan. Insulin signaling is widely identified as the anorexigenic signal for suppression of appetite in animals in satiated conditions (Woods *et al*. 1996). In mammals, insulins are small peptide hormones secreted in the beta cells of pancreatic glands that act in the brain to suppress further consumption of food and stimulate breakdown of glucose via catabolic pathways (Woods *et al*. 1996). With the rise in obesity and other chronic diseases in the western world, there is enormous interest to understand the functioning of this pathway (World Health Organization, 2000). In addition to maintaining the blood sugar level insulin also regulates development such as neuronal proliferation, survival, and neurite outgrowth (Hodge *et al*. 2004, Barres *et al*. 1992, Torres-Aleman *et al*. 1994, Ozdinler and Macklis 2006). In *C. elegans*, the insulin-like pathway is a key determinant for making survival versus growth and/ or reproduction decisions based on the environmental conditions, thus making it a fine example of a signal transduction pathway that regulates developmental plasticity. Insulin-like pathway genes are crucial for making normal dauer formation decisions and are considered to be important for coupling of environmental conditions to genetic developmental programs. Some of the first genes discovered in this pathway were the *daf-2* insulin receptor, *age-1* phosphoinositide 3-kinase, and *daf-16* FoxO transcription factor. Worm strains carrying mutations in *daf-2* and *age-1* were found to

be *daf-c* in response to environmental stress (Riddle *et al*. 1981, Paradis and Ruvkin, 1998, Morris *et al*. 1996, Paradis *et al*. 1999). Mutations in *daf-16,* on the other hand, showed *daf-d* phenotype (Thomas *et al*. 1993, Gottlieb and Ruvkun 1994, Morris *et al*. 1996, Ogg *et al*. 1997, Riddle and Albert 1997, Gems *et al.* 1998). A widely accepted model suggests that *daf-2* and *age-1* function upstream in the insulin pathway such that in favorable conditions, secreted insulin-like proteins (ILPs) bind to DAF-2 receptor and activate AGE-1, leading to production of PIP_3. PIP_3 can in turn stimulate the downstream protein kinase cascade consisting of phosphoionositide dependent kinase PDK-1, and serine/threonine kinases AKT-1, and AKT-2 (Fig. 4) (Morris *et al.* 1996, Morris *et al*.1996, Kimura *et al*. 1997, Paradis and Ruvkun, 1998*,* Paradis *et al*. 1999, Fielenbach and Antebi 2008). AKT-1/2 can in turn phosphorylate DAF-16 FoxO that results in retention of the transcription factor in the cytoplasm in favorable conditions. In unfavorable conditions, DAF-16 is sequestered to the nucleus to positively regulate genes required for dauer formation (Brunet *et al*. 1999, Lee *et al*. 2001, Lin *et al*. 2001). DAF-16 is widely expressed in ectoderm, muscles, intestine, and neurons regulating specialized functions (Lee *et al*. 2003, McElwee *et al*. 2003, Murphy *et al*. 2003). For instance, DAF-16 expression in neuron specific manner is required for dauer formation and expressing *daf-16* in intestine was sufficient for regulating lifespan in animals (Libina *et al*. 2003).

With respect to ILPs, there are about 40 predicted genes that participate in this pathway (Pierce *et al*., 2001). ILPs are small peptide hormones many of which have been investigated for their role in dauer formation and longevity like DAF-28, INS-1, and INS-7, to name a few (Malone and Thomas 1994, Li *et al*. 2003, Murphy *et al*. 2003, Hu

et al. 2007). Amongst all the ILPs, *ins-1* encodes for the ILP most similar to human insulin to an extent that expression of human insulin cDNA can mimic function in *C. elegans* (Hu *et al*. 2007).

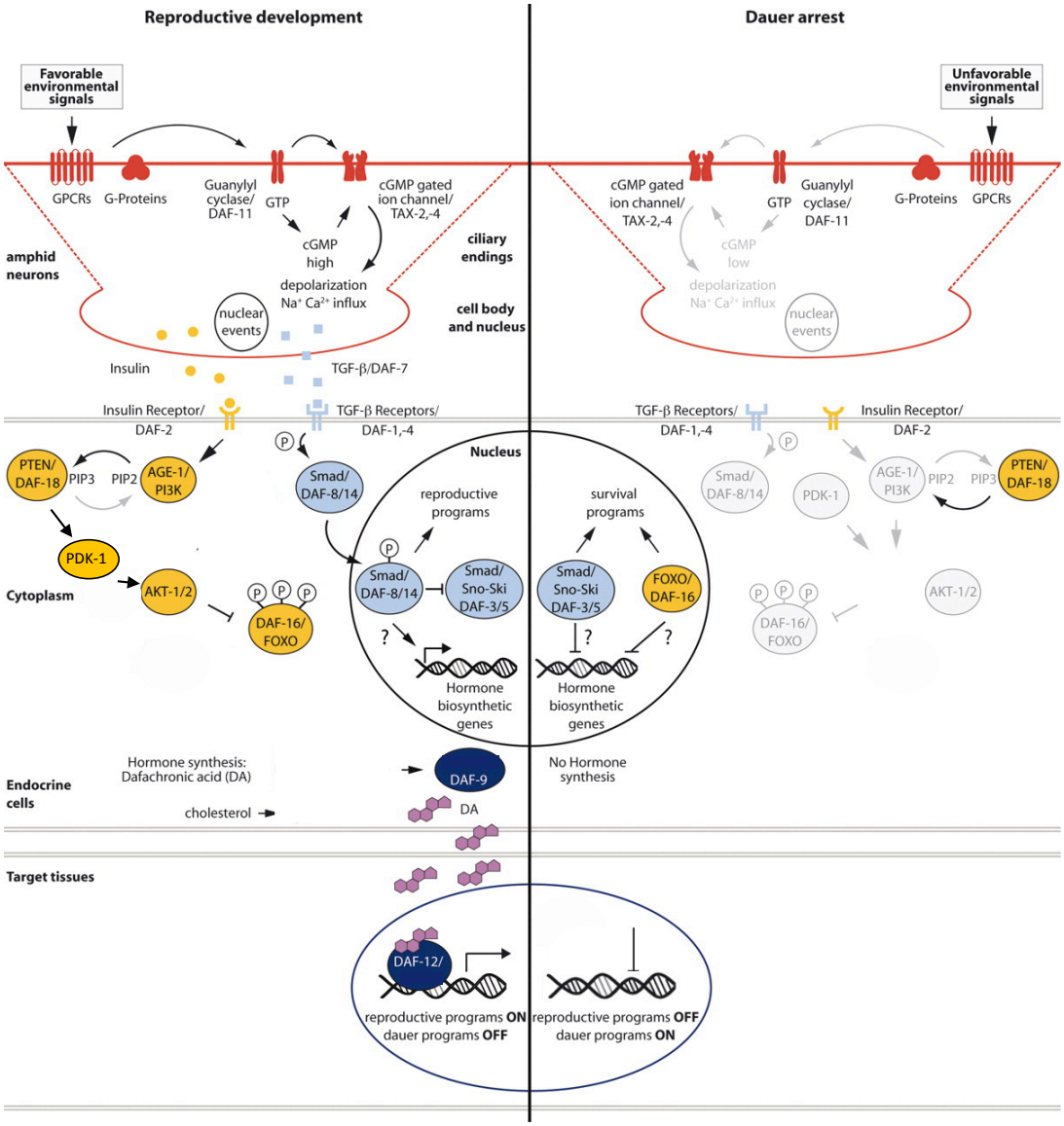


Fig. 4. Sequence of events in reproductive growth and dauer formation in *C. elegans*. The figure above represents the cellular and molecular events in a chronological order in response to favorable and unfavorable environments, including alteration of the signal transduction pathways that are conserved across species. Figure modified from Fielenbach and Antebi 2008.

Most of the ILPs are expressed in neurons, including DAF-28, which is expressed in ASJ and ASI neurons (Pierce *et al*. 2001). Mutation in *daf-28* can result in

downregulation of DAF-2/insulin signaling, consistent with DAF-28 binding DAF-2 (Li *et al*. 2003). Expression of *daf-28* can be inhibited by presence of pheromone or lack of food implying the role of environmental cues in regulation of insulin-like signaling (Li *et al.* 2003). In contrast, deletion of *ins-1* does not exhibit any obvious dauer formation phenotype but is suggested to have an antagonistic effect over insulin-like signaling (Pierce *et al*. 2001). INS-7 on the other hand is suggested to be the agonist of DAF-2 and is upregulated by the insulin-like pathway activity. (Pierce *et al*. 2001, Murphy *et al*. 2003, Husson *et al*. 2007).

Mutations in dauer formation pathway genes, such as *daf-2*, *daf-16*, *age-1* and other PDK/AKT pathway components, can also result in additional phenotypes like longer lifespan and improved stress resistance in *C. elegans* influenced by environmental cues (Kenton *et al*. 1993, Larsen *et al*. 1995, Morris *et al*. 1996, Lin *et al*. 1997, Ogg *et al*. 1997, Kimura *et al*. 1997, Paradis and Ruvkin 1998, Lee *at al*. 2003, McElwee *et al*. 2003, Murphy *et al*. 2003). Individual components like *daf-2* insulin receptor and *age-1* phosphoinositide 3-kinase were found to be orthologous to insulin/IGF-receptors (IGFR) and PI3 Kinase (PI3K) in mammals, respectively (Morris *et al*. 1996, Kimura *et al*. 1997). In a separate study drawing parallels in *C. elegans* and mammals, human FKHRL1 could partly replace *daf-16* FOXO transcription factor proving these genes are orthologous (Lee *et al*. 2001). Studies both in humans and *C. elegans* have indicated that *daf-16* or its orthologs are targets of the insulin-like pathway and are regulated in a similar manner (Lin *et al* 1997, Ogg *et al*. 1997). Given the similarity in the components and mechanisms of insulin regulation between mammals

and worms, the prospect that this knowledge can be extended to humans to increase stress resistance and lifespan is intriguing.

1.4.2 TGF-β like pathway

Transforming growth factor-β (TGF-β) superfamily of ligands are cytokines that include bone morphogenic proteins (BMP), growth and differentiation factor (GDFs), activin/inhibin, and TGF-β. The general sequence of events occurring in this pathway involves binding of TGF-β family of ligand to tyrosine kinase (type I and II) receptors (Reviewed in Derynck and Miyazono 2008). This ligand-receptor interaction stimulates serine/threonine kinase activity of the receptor to phosphorylate downstream SMAD transcription factors. Phosphorylated receptor activated SMADs (R-SMADs) form dimers with common mediator SMADs (co-SMADs) to get sequestered to the nucleus to regulate target genes (Reviewed in Massagué 2000, Attisano and Wrana, 2002).

TGF-β regulates various developmental aspects in animals (Kingsley 1994). In *Drosophila*, TGF-β signaling is essential during dorso-ventral axis formation in early embryos and cell-to-cell signaling for tissue formation in the gut. In *Xenopus*, expression of specific genes of this pathway is required for proper body planning. Mammalian TGF-β also regulates sexual development, pituitary hormone production, and bone and cartilage formation (Kingsley 1994). Misregulation of TGF-β can also lead to cardiovascular, autoimmune and fibrotic diseases (Border and Noble 1997, Lebrin *et al*. 2005, Wan and Flavell 2008). Components of the TGF-β pathway are potential therapeutic targets for treatment of cancer as TGF-β signaling operates as a tumor-suppressing pathway in normal cells and promotes tumor-promoting programs in cancerous cells (Derynck and Akhurst 2007, Massagué 2008).

In *C. elegans*, DAF-7 is one of the five identified TGF-β ligands, which binds to the type I and type II serine/threonine kinase receptor system encoded by *daf-1* and *daf-4* genes, respectively (Georgi *et al*. 1990, Estevez *et al*. 1993, Ren *et al*. 1996, Schackwitz *et al*. 1996, Patterson and Padgett 2000, Gumienny and Savage-Dunn 2005, Wu and Hill 2009) (Fig. 4). DAF-7/ TGF-β like protein is expressed in ASI, ADE, OLQ, and ASJ in favorable environmental conditions (Ren *et al*. 1996, Schackwitz *et al*. 1996, Meisel *et al*. 2014). DAF-7 binds to DAF-1/DAF-4 receptors that phosphorylate the downstream R-SMAD proteins namely, DAF-8, and DAF-14 (Fig. 4). The activated DAF-8 and DAF-14 SMAD then antagonizes co-SMAD DAF-3 function, allowing reproductive programs to be favored (Fig. 4). DAF-5 is a sno/ski acting protein as a co-factor of DAF-3 (Patterson *et al*. 1997, Thatcher *et al*. 1999, Da Graca *et al*. 2004, Massagué *et al*. 2005, Park *et al*. 2010). Both *daf-3* and *daf-5* mutants are *daf-d*, while the rest of the mutants belonging to this pathway are *daf-c,* thus important for normal dauer formation (Patterson and Padgett, 2000, Da Graca *et al*. 2004, Tewari *et al*. 2004). *daf-3* loss-of-function mutants can also exhibit *daf-c* phenotype at 27°C implying a possible partnership of DAF-3 with other SMADs or transcription factors in a temperature dependent manner, acting as an activator or a repressor of the same target genes at different temperatures (Thomas *et al*. 1993, Ailion *et al*. 2000, Attisano and Wrana 2000).

Transforming growth factor-β (TGF-β) is a well-established pathway that is conserved across organisms both at functional and molecular level (Padua and Massagué 2009, Wu and Hill 2009). Dpp and BMP5 are two examples of TGF-β ligand found in *Drosophila* and humans, respectively (Morita *et al*. 1999, Suzuki *et al*. 1999).

sma-2, *sma-3*, and *sma-4* are *C. elegans* SMAD genes that are a conserved family of TGF-β pathway components referred to as 'dwarfins' in *Drosophila* and vertebrates required for cell-to-cell signaling for promoting growth and development (Padgett *et al*. 1987, Sekelsky *et al*. 1995, Savage *et al*. 1996). In *C. elegans*, these SMADs are required for body size regulation and maintenance of reproductive tissues (Luo *et al*. 2009, Luo *et al*. 2010, Wang *et al*. 2005). Also, DAF-3 is similar to the Medea protein in *Drosophila* and the vertebrate Smad 4 (Raftery *et al*. 1995, Hahn *et al*. 1996, Lagna *et al*. 1996, Savage *et al*. 1996, Wisotzkey *et al*. 1998). Further, interactions between DAF-3 and DAF-5 are very similar to that seen between their human counterparts (Da Graca *et al*. 2004, Tewari *et al*. 2004). Understanding of the TGF-β signaling in *C. elegans* is invaluable for getting insight into functioning of this pathway in other animal systems and applying our knowledge to identify targets associated with various diseases caused due to the misregulation of TGF-β.

1.4.3 Steroid hormone pathway

Steroid hormones are important for regulating development and physiology and are derivatives of cholesterol (Berg *et al*. 2012). In animals, steroids comprise a large group of hormones including sex hormones, glucocorticoids, and neurosteroids (Barnes 2006, Reddy 2010, Ruiz- Cortés 2012). In insects, steroids are synthesized for growth, development, and molting (Bollenbacher *et al*. 1975). In plants, hormones such as the brassinosteroids are required for growth and cell differentiation (Wang and Chory 2000). A previously proposed model for steroid signaling in animals suggested the requirement of intracellular fusion of steroid hormones with their respective nuclear receptors for transcription of target genes (Riddiford *et al*. 2003). More recent findings have shown

binding of steroids to cell surface receptors, specifically G protein coupled receptors (GPCR) that activate Ca^{2+} influx for cGMP signaling (Details in Section 1.3.4), to bring about rapid responses (Lösel *et al*. 2003, Wang *et al*. 2015) in humans (Maggiolini and Picard 2010), *Drosophila* (Srivastava *et al.* 2005), *Bombyx mori* (silkworm)(Elmogy *et al*. 2004), and *Helicoverpa armigera* (Cotton bollworm)(Liu *et al*. 2011). These examples demonstrate the mechanistic similarities across animals in terms of steroid hormone regulation.

Utilizing the simple and powerful genetics of *C. elegans* model system and the information available regarding the steroid signaling pathway, there has been tremendous progress made to understand the role of hormone signaling at cellular and organismic level, and provides the scope to learn much more about the complex biological processes that cannot be understood using mammalian systems. In *C. elegans*, insulin-like and TGF-β pathways converge onto the steroid signal transduction pathway (Riddle *et al*. 1981, Vowels and Thomas 1992, Thomas *et al*. 1993) (Fig. 4). Environmental cues favoring reproduction allow the synthesis or availability of steroid hormones with help of DAF-9 enzyme required for steroid biosynthesis. DAF-9 functions non-cell autonomously in epidermis, hermaphrodite spermatheca, and XXX endocrine cells. XXX cells act as a junction to integrate inputs from upstream signal transduction pathways that stimulate production of dafachronic acid (DA) (Gerisch and Antebi 2004, Li *et al*. 2004, Hu *et al*. 2006). Additionally, XXX neuron has also been shown to express genes like *sdf-5*, *eak-4* and *eak-6* required for promoting DAF-9 activity and steroid hormone biosynthesis (Ohkura *et al*. 2003, Hu *et al*. 2006). The hormones then serve as ligands for the nuclear hormone receptor (NHR) DAF-12 (Fig. 4). An earlier

study proposed need of two steroid dafachronic acids (Δ^4 and Δ^7) as endogenous ligands to DAF-12 to promote development of worms from L2 to L3 stage (Motola *et al*. 2006, Antebi *et al*. 2000, Snow and Larsen 2000). A more recent study conducted comparative metabolomics to identify other DAF-12 ligands, as the current understanding of DA function does not explain all roles of DAF-12 (Patel *et al*. 2008, Williams *et al*. 2010, Wollam *et al*. 2012). The new study showed presence of additional endogenous ligands, $\Delta^{1,7}$-DA being the most abundant aside from smaller amounts of already known Δ^7-DA and 3α-OH-Δ^7-DA without any trace of DA- Δ^4 as thought to be found earlier (Mahanti *et al*. 2014). It is suggested that DAF-12 integrates signals from the upstream signal transduction pathways and ensures an appropriate regulatory switch between dauer formation and continuous reproductive development besides governing metabolism, lifespan and other aspects of *C. elegans* life history traits (Riddle *et al*. 1981, Albert and Riddle, 1988, Thomas *et al*. 1993 Gerisch *et al*. 2001, Jia *et al*. 2002). DAF-12 is widely expressed and is localized in the nucleus (Gerisch and Antebi 2004, Mak and Ruvkun 2004).

Mutation in *daf-12* results in either *daf-c* or *daf-d* phenotype depending on the allele being tested. This variability in *daf* phenotype suggested two separable gene activities implying that DAF-12 is important for promoting development into L3 stage in favorable conditions as well as promoting dauer programs in stressful environments (Antebi *et al*. 1998). However, *daf-9* mutants are unconditionally *daf-c* (Gerisch *et al*. 2001, Jia *et al*. 2002).

The two important components *C. elegans* steroid hormone pathway, DAF-12 and DAF-9, also have homologs in mammals and flies. DAF-12 is the homolog of

mammalian Vitamin D and liver-X receptors called VDR and LXR respectively (Bento *et al*. 2010, Ogawa *et al*. 2011 and Sommer and Ogawa, 2011). DAF-9 has sequence similarities in *Drosophila* CYP18 gene, and human CYP17 and CYP21 (Nelson 1998) that carry out important functions of cholesterol, steroid and other lipid biosynthesis (Hall 1986, Miller 2002, Mellon and Griffin 2002, Rainey *et al*. 2002).

1.4.4 cGMP signaling

The role of cyclic GMP pathway has been elucidated in diverse biological processes varying from modulating cell physiological changes in cardiovascular system to phototransduction in the retina of animals given the wide range of ligands triggering this pathway (reviewed in Potter *et al*. 2011). cGMP are secondary messengers in hormone signaling where the hormone itself is the first messenger (Sutherland 1972, Rodbell 1995). With respect to medical applications, genes functioning in the cGMP signaling are considered to be potential targets for drug design for treatment of various conditions, including breast cancer (Windham and Tinsley 2015) and schizophrenia (Shim *et al*. 2016).

In *C. elegans*, the cGMP pathway contributes to dauer formation by allowing animals to detect environmental conditions (Fig. 4). Transmembrane guanynyl cyclase, encoded by *daf-11,* alters the functioning of the ion gate channels encoded by *tax-2* and *tax-4* in response to the external environmental stimuli. In stress free environment, DAF-11 catalyzes conversion of GTP to cGMP that in turn facilitates intracellular Ca^{2+} influx via TAX-2 and TAX-4 ion channels to suppress dauer formation (Komatsu *et al*. 1996, Coburn *et al*. 1998, Birnby *et al*. 2000) (Fig. 4). DAF-11, TAX-2, and TAX-4 are localized in the cilium of a subset of amphid neurons and are thought to function upstream of

insulin and TGF- β pathways to contribute to dauer formation decisions (Birnby *et al*. 2000, Coburn and Bargmann, 1996; Li *et al*., 2003; Murakami *et al*., 2001) (Fig. 4). An additional component of this pathway, DAF-21, which is an Hsp90 homolog was found to be functioning in a similar loci as DAF-11 in the genetic pathways and is thought to be a regulator of DAF-11 function (Thomas *et al*. 1993, Vowels and Thomas 1992, 1994, Birnby *et al*. 2000).

1.5 RNAi pathways are a complex gene regulatory mechanism in *C. elegans*

RNA interference (RNAi) is a mechanism conserved across species required to regulate expression of coding genes, transposons, pseudogenes, and other non-coding small RNAs (Billi *et al*. 2014). In worms, small interfering RNAs (siRNAs) are characterized by their biogenesis and associated argonautes (AGOs). Primary siRNAs are lowly abundant, have Dicer-dependent biogenesis, and are 26 nucleotides long with a 5' guanine (26G-siRNAs) (Bernstein *et al*. 2001, Grishok *et al*. 2001, Ketting *et al.* 2001, Knight and Bass 2001, Han *et al.* 2009, Pavelec *et al*. 2009, Vasale *et al*. 2010). Through an unknown mechanism, 26G-siRNAs stimulate the production of highly abundant siRNAs that are 22 nucleotides long with a 5' guanine (22G siRNAs/secondary siRNA) and are synthesized through the action of RNA dependent RNA polymerases (RdRPs) (Smardon *et al*. 2000, Ketting *et al.* 2001, Knight and Bass 2001, Simmer *et al*. 2002, Ambros *et al*. 2003, Vought *et al.* 2005, Maine *et al*. 2005, Aoki *et al.* 2007, Pak and Fire 2007, She *et al*. 2009, Vasale *et al.* 2010, Gent *et al.* 2010, Pak *et al*. 2012). In addition, a group of proteins called the *Mutators* have been shown to play a role in siRNA amplification of both 26G- and 22G-siRNAs classes

(Zhang *et al.* 2011, Phillips *et al*. 2012). Specific small RNA classes bind to one or more of the 26 AGO proteins in *C. elegans*, which are characterized by their expression patterns and whether they function in the cytoplasm or nucleus (Yigit *et al.* 2006). Although the biogenesis of endogenous siRNAs has been relatively well characterized, we know little about how RNAi pathways target and regulate endogenous genes.

1.5.1 CSR-1 AGO pathway

In the germ line, the CSR-1 pathway requires CSR-1 AGO, RdRP EGO-1, helicase DRH-3, and EKL-1 (a tudor domain protein) to target genes for transcriptional regulation important for germline development. For example, EGO-1 is important for gametogenesis and heterochromatin assembly of unpaired chromosomes in meiosis (Qiao *et al*. 1995, Smardon *et. al* 2000, Vought *et al*. 2005, Maine *et al*. 2005). Helicase DRH-3 is also important for chromosome segregation and germ cell proliferation (Aoki *et al*. 2007, Eki *et al*. 2007, Rocheleau *et al*. 2008, Nakamura *et al*. 2007, Tabara *et al*. 2002, Duchaine *et al*. 2006, She *et al*. 2009). Additionally, EKL-1 is important for gamete production, embryo development, chromosome segregation, and fertility (Robert *et al*. 2005, Rocheleau *et al*. 2008, Gu *et al*. 2009, Claycomb *et al*. 2009, She *et al*. 2009). CSR-1 AGO was recently shown to possess the slicer activity *in vivo* to downregulate maternally expressed genes necessary for proper embryonic cell divisions (Gerson-Gurwitz *et al*. 2016). CSR-1 has also been shown to mediate proper compaction of the holocentric chromosomes in *C. elegans* and hence, animals carrying a null mutation in *csr-1* are sterile due to chromosomes failing to segregate properly in the germline (Claycomb *et al*. 2009). Also, CSR-1 was shown to be residing in the P-granule and localized to the nucleus in the oocytes (Claycomb *et al*. 2009). In one

study, 22G small RNAs associated with CSR-1 are antisense to genes expressing in the germline but a majority of them do not cause downregulation of gene expression of their targets (Claycomb *et al*. 2009). In a different study, CSR-1 was shown to be directly and positively regulating histone expression and the failure of chromosomes to segregate in mutants, attributed to misprocessed histone mRNA (Avgousti *et al*. 2012). More recent studies have implied CSR-1 to be positively regulating genes of both germline and somatic cells (Hall *et al*. 2013, Seth *et al*. 2013, Wedeles *et al*. 2013, Cecere *et al*. 2014). CSR-1 was also found to be involved in translational repression of target mRNA (Friend *et al*. 2012). In addition, CSR-1 has been shown to play an important role in accumulation of H3K9me2 marks on unpaired X chromosomes, implying a role of small RNA-mediated pathway during meiosis (She *et al*. 2009). Previous work from our lab has also shown that CSR-1 is important for changes in chromatin state in postdauers compared to control animals (Hall *et al*. 2013). Furthermore, comparative functional genomic analysis between *C. elegans* and *C. briggsae* found that CSR-1 has a conserved role in regulating germline protein coding genes in the germline (Tu *et al*. 2014). While CSR-1 has been implicated in important gene regulation mechanisms in *C. elegans* germline, their role in somatic cells needs more investigation (Yigit *et al*. 2006, Claycomb *et al*. 2009).

1.5.2 *Mutator* loci

The *Mutator* proteins play an important role in siRNA amplification, including of those that bind to CSR-1 (Claycomb *et al*. 2009, Phillips *et al*. 2012). One of the *Mutators*, MUT-16 (Q/N rich domain protein), is required for the formation and localization of a protein complex referred to as "*Mutator* focus" in the germline – an RNA

processing compartment where MUT-16 is involved in amplification of siRNA and subsequent silencing of mRNA targets by participating in the 22G siRNA pathway (Phillips *et al.* 2012). The other *Mutators* that are part of this complex are MUT-2 (nulceotidyl transferase), MUT-7 (3'-5' exonuclease), MUT-8/RDE-2 (unknown function), MUT-14 (Dead-box RNA helicase), MUT-15 (unknown function), and RRF-1 (RdRP). However, it is not clear if such a complex exists in the somatic tissue. The majority of the amplified 22G-siRNA are not destined to be part of CSR-1 pathway and are escorted by worm specific argonaute (WAGO) to gene targets including transposons and aberrant transcripts to bring about silencing. MUT-16 has been implicated in both exogenous and endogenous RNAi pathways. Apart from failing to silence transposons in the germ line, *mut-16* mutants exhibit "increased incidence of male" phenotype, due to increased chromosome nondisjunction events (Ketting *et al*. 1999, Vastenhouw *et al*. 2003). To further assess the role of MUT-16 specifically in endogenous RNAi pathway in *C. elegans*, Zhang *et al.* (2011) found that MUT-16 is also required for 26G siRNA and 22G siRNA formation in somatic tissues to target coding genes by transcriptional (via nuclear RNAi pathway) and post-transcriptional silencing (Zhang *et al*. 2011). Published data has shown that siRNAs antisense to insulin-like signaling and TGF-β pathway genes require CSR-1 and MUT-16 for biogenesis and may be potential targets of these pathways (Claycomb *et al*. 2009, Phillips *et al*. 2012). Some of these genes include *daf-2* insulin receptor, *daf-16* FOXO, *daf-1* and *daf-4* TGF-β receptors, *daf-3* SMAD, and *daf-5* SNO/SKI (Claycomb *et al*. 2009, Phillips *et al*. 2012).

The current model suggests that siRNA are correctly channeled to their destinations via *Mutator* complex and the adjacent P granules that use separate

machineries as described above in order to (a) silence aberrant transcripts including transposons, (b) regulate transcriptional gene regulation where the siRNA may be "protecting" transcriptionally active genes by defining euchromatin regions, respectively (Zhang *et al*. 2011, Phillips *et al*. 2012).

In this dissertation, we investigated the role of *Mutators* and CSR-1 AGO pathway components in regulation of genes in dauer formation. First, we identified RNAi pathway required for dauer formation in response to high amounts of pheromone (Chapter 1). We were also able to identify the possible tissue where RNAi pathways might potentially function to mediate dauer formation. Furthermore, we propose a model that explains an underlying mechanism of how RNAi may be involved in the dauer developmental decision in the presence of high pheromone concentration (Chapter 1). Additionally, we were able to develop specialized assays to test if the results from Chapter 1 were consistent across stressful condtions, *i.e*, in high temperature and starvation conditions (Chapters 3 and 4, respectively). In Chapter 5 we investigate a potential role of RNAi pathways in regulation of dauer exit decision.

Chapter 2

RNAi pathway is required for pheromone induced dauer formation

2.1 Synopsis

Routine experiments conducted in our lab involve subjecting worms to exogenous pheromone to obtain dauers for downstream applications. In this process, our lab has identified a subset of RNAi pathway mutants exhibiting *daf-d* or *daf-c* phenotypes when subjected to high concentrations of pheromone. This observation led us to form our first hypothesis that the *Mutators* and CSR-1 AGO are required for dauer formation in *C. elegans* in response to pheromone stress, which we verified using more sensitive dauer formation assays with high pheromone concentrations. Next, we performed epistasis experiments using RNAi and TGF-β or insulin-like signaling mutant strains to show that *Mutator* MUT-16 functions upstream of or in parallel to these evolutionarily conserved dauer formation pathways. We hypothesized that *Mutators* and the CSR-1 pathway are functioning in sensory neurons to regulate the dauer decision, since the detection of environmental stresses and the resulting differential regulation of TGF-β and insulin signaling pathways occurs in neurons (Golden and Riddle 1982, Golden and Riddle 1984a, Ren *et al*. 1996, Li *et al*. 2003). Consistent with our hypothesis, we showed that a strain expressing a *mut-16::gfp* translational fusion driven by a pan-neuronal promoter (*rab-3*) in the *mut-16*(*pk710*) background, exhibited wild-type dauer formation phenotype in response to high pheromone conditions. Furthermore, by rescuing MUT-16 in specific neurons, we showed that a subset of

pheromone-sensing neurons ASI, ADL, and ASJ rescued the *daf-d* phenotype of *mut-16*(*pk710*). Finally, we show that G protein coding genes *gpa-1*, *gpa*-3, and *gpc*-1 are downregulated in *mut-16*(*pk710*) and *csr-1* hypomorph backgrounds. We propose a model where the CSR-1 RNAi pathway is required in neurons to positively regulate G protein expression to facilitate detection of pheromone to induce dauer developmental decision in early larval stages.

2.2 Methods

2.2.1 Cloning and generation of rescue strains and double mutants

Genomic DNA template was used to amplify the full-length *mut-16* gene and all the promoters described in this study. Upstream regulatory regions were amplified for tissue specific or neuron-specific rescues using the following genes: *rab-3* (pan-neuronal), *trx-1* (ASJ), *unc-130* (ASG), *gpa-4* (ASI), *sre-1* (ADL), *srh-142* (ADF), *odr-10* (AWA), and *ges-1* (intestine) (Egan *et al*. 1995, Troemel *et al*. 1995, Sengupta *et al*. 1996, Nonet *et al*. 1997, Jansen *et al*. 1999, Sagasti *et al*. 1999, Sarafi-Reinach and Sengupta, 2000, Marshall and McGhee, 2001, Lanjuin *et al*. 2003, Miranda-Vizuete *et al*. 2006,) (Appendix 2, contains list of strains used in this study).

The genomic *mut-16* gene was fused to tissue-specific promoters and the *gfp* gene using fusion PCR (Hobert 2002). ADL- and ASI-specific *mut-16* rescue constructs were described previously (Sims *et al*. 2016). *rab-3*p::*mut-16*::*gfp, trx-1*p::*mut-16*::*gfp, unc-130*p::*mut-16*::*gfp, gpa- 4*p::*mut-16*::*gfp,* and *sre-1*p::*mut-16*::*gfp* were cloned into the TOPO-XL (Life Technologies) and injected into *mut-16*(*pk710*) at a concentration of 8 ng/µL. For *srh-142*p::*mut-16*::*gfp, odr-10*p::*mut-16*::*gfp,* and *ges-1*p::*mut-16*::*gfp*, purified PCR products were directly injected into *mut-16*(*pk710*) at concentrations of 8

ng/μL, 4 ng/μL, and 1 ng/uL, respectively. *unc-122*p::*dsRed* (30 ng/μL) was used as the co-injection marker. Two independent transgenic lines were used in all dauer formation assays.

mut-16(*pk710*); *csr-1*(*tm892*) double mutants used in the high pheromone dauer formation assays were generated by Dr. Maria Ow (Syracuse University). Double mutants for epistasis analysis were generated by crossing *mut-16*(*pk710*)(♂) and *daf-2*(*e1370*)(♀), and *mut-16*(*pk710*)(♂) and *daf-7*(*e1372*)(♀) to obtain *mut-16*(*pk710*)*; daf-2*(*e1370*) and *mut-16*(*pk710*)*; daf-7*(*e1372*), in the F_2 population.

2.2.2 Pheromone dauer formation assay

To test dauer formation in the presence of high pheromone concentrations, we conducted assays as previously described using crude pheromone preparations (Zhang *et al.* 2013) with some modifications (Neal *et al.* 2015). The modifications were incorporated in order to accommodate for the severe sterility phenotypes of the mutants we tested.

Plate preparation: For each independent batch of crude pheromone, we calculated the amount of pheromone that resulted in 33% dauers in wild-type animals, which is defined as 1 activity unit (Zhang *et al.* 2013). Fig. 5 represents the activity curve for one of the batches of crude pheromone used for the assays. Each dauer formation plate contained 4 activity units of pheromone that was mixed in the media during preparation of the plates. Water was added instead of pheromone for the control plates. Each assay plate was seeded with 20 μL of 8mg/mL (0.16 mg) heat-killed *E. coli* OP50.

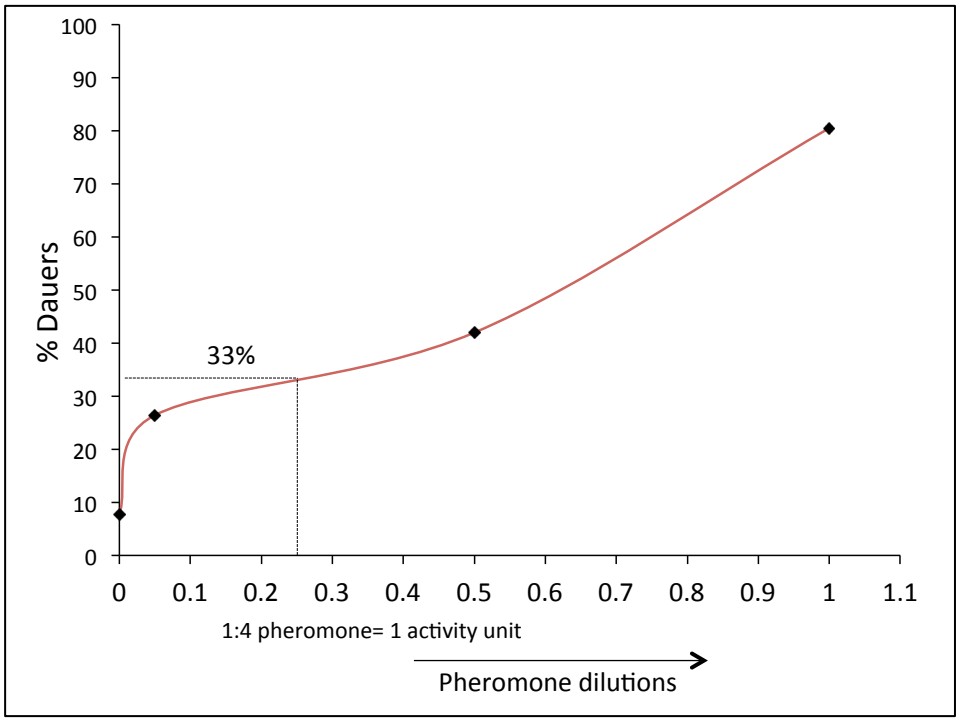


Fig. 5. An example for calculation of activity units for a batch of crude pheromone. The above graph was plotted for one of the batches of crude pheromone extracted in our lab to calculate the number of activity units. 1 activity unit of pheromone is defined as the amount of pheromone required to obtain for 33% dauers.

Egg-laying: 5 well-fed adult worms (egg-laying, 48 hours after L4) were allowed to lay eggs on assay plates for 3-6 hours at room temperature. The worms were removed when approximately 60-80 eggs were laid on each of the plates. Since many of the mutant strains (*mut-2(ne298)*, *mut-7*(*pk720*), *csr-1*(*hypomorph*), and *drh-3*(*ne2453*)) exhibited sterility phenotypes, additional adults (3-4) were transferred to expedite the egg laying on mutant plates. However, egg-laying was not allowed to continue for more than 12 hours. To ensure that these adults did not starve due to extended times on plates, the amount of food was doubled when testing *mut-2(ne298), mut-7*(*pk720*), *csr-1* hypomorph, and *drh-3(ne2453)*. We did not observe a significant change in dauer formation for wild-type animals upon addition of excess food (Student's t-test, $p = 0.17$).

We made an exception when testing for the percentage of dauers for *csr-1(tm892)* and *mut-16(pk710)*; *csr-1(tm892)*, where we allowed 8 adults to lay eggs on 6 plates (usually done in duplicates when testing other strains), owing to the presence of the balancer that further reduced the incidence of *csr-1*(*tm892*) mutants in the progeny. To avoid OP50 contamination on assay plates, adults were allowed to crawl on unseeded NGM plates for 20 minutes prior to transferring them onto assay plates. In severe contamination cases adults were washed with streptomycin (50 µg/ml) dissolved in M9 buffer. The assay plates were shifted to 25°C incubator after sufficient eggs were laid (60-80 eggs).

Scoring dauer formation: The percentage of dauer formation on the assay plates was scored after 3 days. In case of mutants that exhibited increased number of partial dauers, we waited an additional day to confirm the results. Dauers were distinguished from adults by examining the pharynx activity and formation of the alae (Cassada and Russel, 1975, Popham and Webster 1979, Albert and Riddle, 1988, Riddle and Albert 1997). The progeny for all the genotypes were scored at the same time. In addition to dauers and non-dauers (adults/L4s), we also counted an additional category of larvae exhibiting the partial dauer phenotype. Partial dauers showed intermediate phenotype between dauer and non-dauer phenotypes displaying reduced pharyngeal pumping and lesser fat accumulation compared to dauers resulting in thinner cuticle (Cassada and Russell, 1975, Popham and Webster 1979, Albert and Riddle, 1988, Riddle and Albert 1997).

All dauer formation assays were performed at least 3 times with biologically independent samples of each strain. Statistical significance of data was determined

using one-way ANOVA with LSD or Tukey's HSD post hoc tests using SPSS (version 23) software.

2.2.3 RNA preparation and quantitative RT-PCR

Well-fed adult hermaphrodites were bleached according to the protocol described in Stiernagle 2006. Three biologically independent populations of L1 larvae were collected from wild-type, *csr-1* hypomorph, and *mut-16*(*pk710*) strains. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the company protocol and reverse transcribed into cDNA using SuperScript IV Reverse Transcriptase (Invitrogen). Quantitative PCR was performed in triplicate for each reaction according to the protocol for iTaq Universal SYBR Green Supermix (Invitrogen). Normalized gene expression ratios were calculated for the candidate genes *flp-21*, *gpa-1*, *gpa-3*, and *gpc-1* using the mRNA levels of *y45f10d.4*, a somatically expressed gene that is not a CSR-1 target (Claycomb *et al*. 2009) and does not experience gene expression changes due to dauer-inducing conditions (M. C. Ow and S. E. Hall, personal communication). Primers sequences are listed in Appendix 2.

2.2.4 DiI staining

DiI is a red fluroscent stain taken up by the ciliary endings of environmentally exposed amphid neurons including ADL, ASI, ASJ, and ASK implicated in dauer formation (Shaham *et al*. 2006). A plate each of well-fed wild-type and *mut-16*(*pk710*) young adults were transferred into 1.5 mL microcentrifuge tubes using M9 and centrifuged to obtain a pellet. The worms were then resuspended in M9 containing DiI (1:200 dilution) prepared from 2mg/mL stock solution of the dye. The tubes were covered with aluminum foil and incubated at room temperature on a slow rotator for 30

minutes. The worms were then placed on 2% agar pads, paralyzed with sodium azide (100mM), and imaged using Leica DM5500 B and ORCA-R2 Digital C10600 camera by Hamamatsu.

2.3 Results

2.3.1 Endogenous RNAi pathways are required for dauer formation

In order to characterize how RNAi pathways contribute to dauer formation in response to environmental stress, we carried out high pheromone dauer formation assays using strains carrying mutations in a subset of genes with functions in endogenous RNAi pathways. Newly hatched larvae were subjected to high concentrations of crude dauer pheromone and the percentage of animals that formed dauers was calculated (Neal *et al*. 2013).

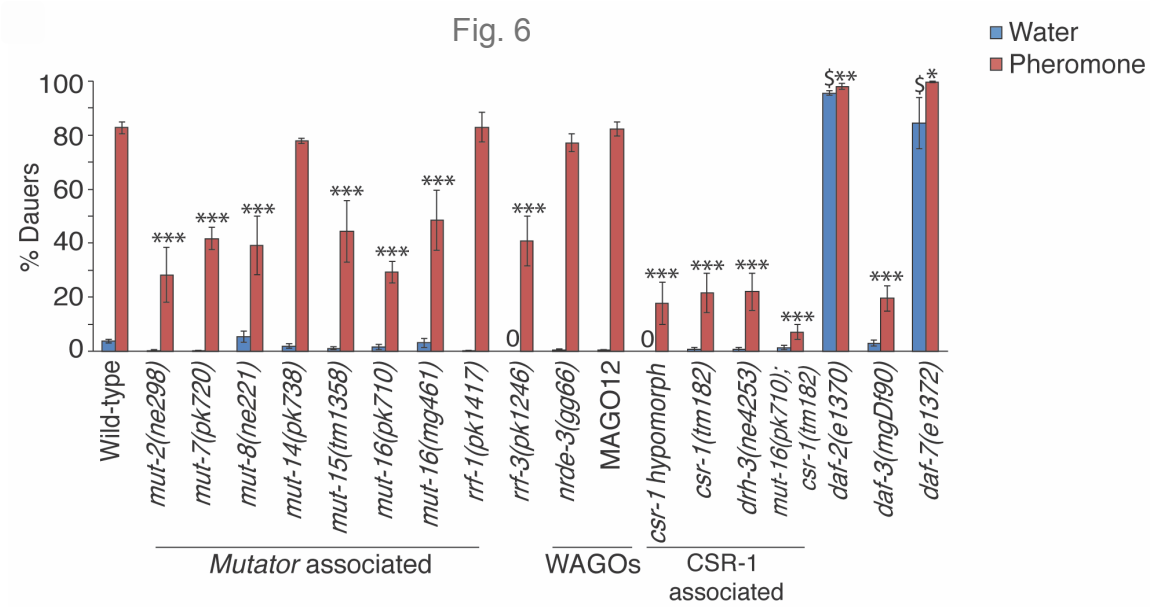
Wild-type larvae populations formed significantly more dauers in high pheromone conditions (82.7 ± 2.2%) compared to control water plates (3.6 ± 0.7%), as expected (Fig. 6). As controls, we also verified that strains with mutations in TGF-β and insulin-like signaling pathways exhibited previously characterized *daf-d* (*daf-3*(*mgDf90*)) and *daf-c* (*daf-7*(*e1372*)*, daf-2*(*e1370*)) phenotypes (Riddle *et al.* 1981, Vowels and Thomas 1992, Gottlieb and Ruvkun 1994). Next, we examined dauer formation phenotypes of strains carrying mutations in genes encoding proteins associated with *Mutator* foci. In the germline, *Mutator* proteins form foci that are dependent on MUT-16 and localize adjacent to P granules (Phillips *et al.* 2012). We observed that mutations in a majority of the *Mutator* genes, *mut-2(ne298)*, *mut-7(pk720)*, *mut-8/rde-2(ne221*), *mut-15(tm1358),* and two alleles of *mut-16(pk710* and *mg461)*, resulted in significantly fewer dauers compared to wild-type in the presence of pheromone (Fig. 6). The *pk710* allele is a null

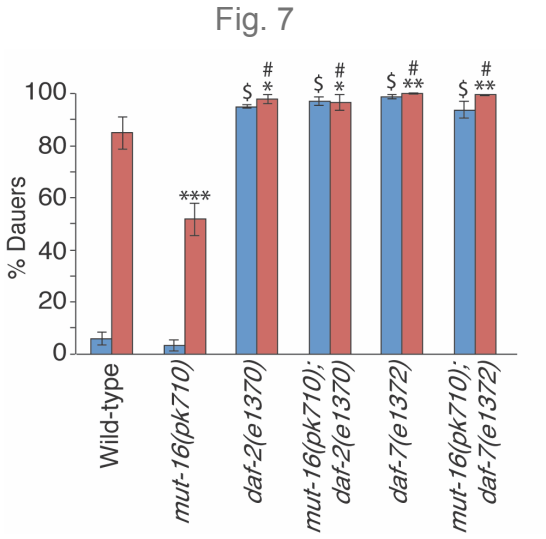
mutation located within the coding sequence of *mut-16*, while the *mg461* allele has a small deletion in the upstream regulatory sequences that disrupts somatic RNAi (Zhang *et al.* 2011). The *mut-16(pk710)* strain exhibited a *daf-d* phenotype that is comparable to the control *daf-3*(*mgDf90*), whereas the other strains, including *mut-16(mg461)*, exhibited intermediate *daf-d* phenotypes (Fig. 6). However, strains with mutations in *mut-14(pk738)* and the *Mutator*-associated RdRP, *rrf-1*(*pk1417*)*,* formed dauers comparable to wild-type levels, indicating that these proteins are not required for dauer formation in response to high pheromone stress (Fig. 6). Interestingly, we found that mutation in another somatic RdRP gene, *rrf-3*, although not identified previously as a part of the *Mutator* complex, exhibited *daf-d* phenotype indicating possible role of RRF-3 in dauer formation. These results indicate that a majority of the *Mutator* proteins and RdRP RRF-3, which are required for siRNA amplification, are necessary for dauer formation in response to high pheromone concentrations.

Next, we asked which endogenous RNAi pathway is required for dauer formation by examining the *daf* phenotypes of strains with mutations in various AGO genes. Since dauer formation occurs during early larval stages, we tested AGOs that are expressed in somatic tissue throughout *C. elegans* development. Previous work has shown that mutations in *Mutator* genes drastically reduce the abundance of siRNAs that associate with worm-specific AGO proteins (WAGOs), including the nuclear AGO NRDE-3 (Zhang *et al.* 2011). Thus, we first tested whether *nrde-3*(*gg66*) and MAGO12 (carrying mutations in all twelve *wago* genes) mutant strains exhibited *daf* phenotypes in response to high pheromone levels. We found that both strains formed dauers similar to wild-type, indicating that *Mutator*-amplified siRNAs required for dauer formation are not

associated with the WAGO RNAi pathways (Fig. 6).

Next, we tested whether the nuclear AGO CSR-1 pathway was playing a role in dauer formation. CSR-1 associated 22G-siRNAs are only slightly reduced for a subset of target genes in a *mut-16*(*pk710*) strain (Zhang *et al.* 2011). Surprisingly, both a null mutant *csr-1*(*tm892*) and a *csr-1* hypomorph strain that expresses CSR-1 only in the germ line (Claycomb *et al.* 2009) exhibited significantly decreased dauer formation levels, similar to the levels of *mut-16*(*pk710*) and *daf-3*(*mgDf90*) strains (Fig. 6). In the germ line, the CSR-1 pathway requires RdRP EGO-1, Dicer-related helicase DRH-3, and Tudor-domain protein EKL-1; however, CSR-1, DRH-3, and EKL-1 are also expressed in somatic tissue during all larval stages (Claycomb *et al.* 2009). We found that the *drh-3(ne4253)* mutant strain also exhibited significant *daf-d* phenotype consistent with that seen in the *csr-1* hypomorph strain in response to high concentrations of pheromone (Fig. 6). We were unable to test an *ekl-1(ok1197)* allele mutant strain due to its sterility phenotype. Finally, in order to determine if CSR-1 and *Mutators* function are required in the same pathway for dauer formation, we subjected *mut-16*(*pk710*);*csr-1*(*tm892*) double mutant to high pheromone concentration. The double mutants exhibited a number of dauers not significantly different from individual *csr-1* mutants (one-way ANOVA with Tukey HSD post hoc test, $p = 1.0$) indicating their roles in the same pathway (Fig 6). These results suggest that *Mutator*-amplified siRNAs associating with the CSR-1 pathway are required for dauer formation in response to high pheromone conditions.





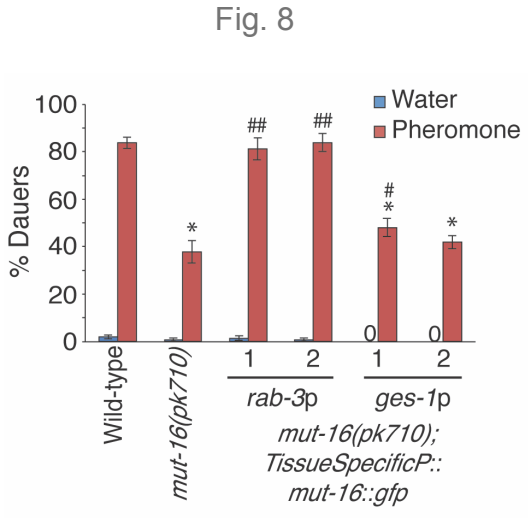


Fig. 6. Proportion of animals forming dauers in response to high pheromone conditions or water plates is shown. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to wild-type on pheromone plates, $ indicates $p < 0.0005$ compared to wild-type on water plates, one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 217 animals. **Fig. 7**. Epistasis analysis was performed using dauer formation assays in high pheromone conditions with *mut-16*(*pk710*), *daf-2*(*e1370*), *daf-7*(*e1372*), *mut-16*(*pk710*)*; daf-2(e1370)*, and *mut-16*(*pk710*)*; daf-7*(*e1372*) strains. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to wild-type on pheromone plates. # indicates $p < 0.0005$ compared to *mut-16*(*pk710*) on pheromone plates. $ indicates $p < 0.0005$ compared to wild-type on water plates, one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 274 animals. **Fig. 8.** Proportion of animals forming dauers in response to high pheromone and water plates is shown for pan-neuronal (*rab-3*p) and intestinal (*ges-1*p) *mut-16* rescue strains. * indicates $p < 0.0005$ compared to wild-type on pheromone plates; # and ## indicate $p < 0.05$ and $p < 0.0005$ compared to *mut-16*(*pk710*), respectively, on pheromone plates; one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 213 animals. All error bars represent S.E.M.

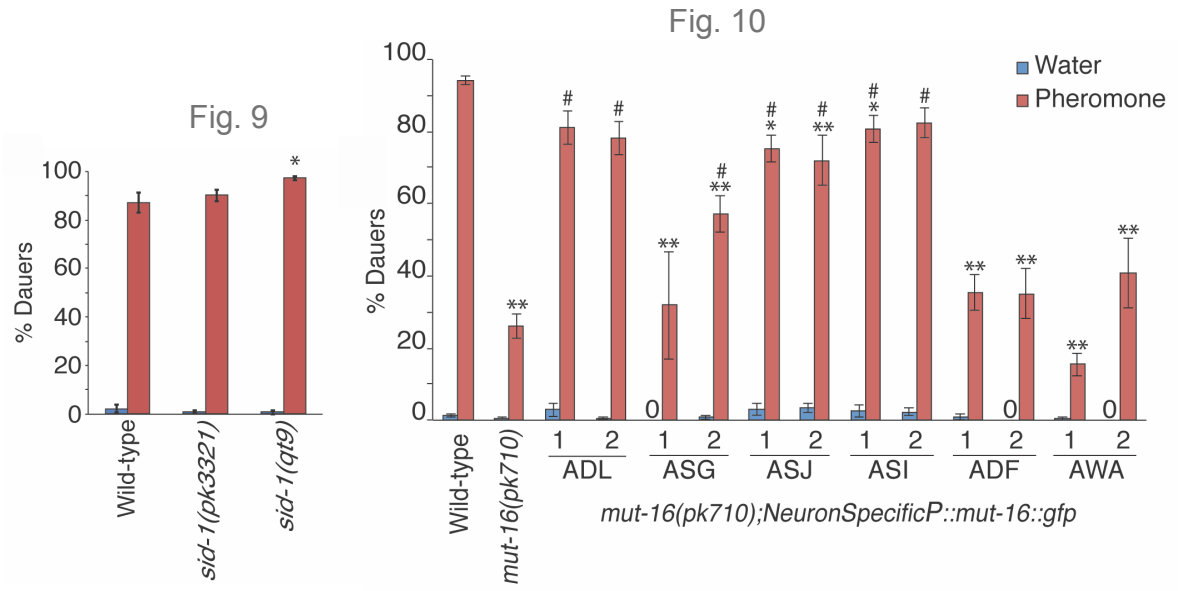


Fig. 9. Proportion of *sid-1* animals forming dauers in response to pheromone. * $p < 0.05$, one-way ANOVA with LSD post hoc test. N = 3 trials; n ≥ 480 animals. **Fig. 10**. Proportion of animals forming dauers in response to high pheromone and water control is shown for wild-type, *mut-16*(*pk710*), and neuron-specific rescue strains. N ≥ 3 trials; n ≥ 173 animals. * $p < 0.05$, ** $p < 0.0005$ compared to wild-type; # $p < 0.0005$ compared to *mut-16*(*pk710*); one-way ANOVA with Tukey's HSD post hoc test. All error bars represent S.E.M.

2.3.2 MUT-16 functions upstream of or parallel to DAF-7 TGF-β and DAF-2 insulin receptor for dauer formation

To address the question of how endogenous RNAi pathways are required for dauer formation, we performed epistasis experiments using RNAi, TGF-β, and insulin-like signaling mutant strains. Prior studies have ordered genes in the TGF-β and insulin-like signaling dauer formation pathways through epistasis analysis using *daf-d* and *daf-c* phenotypes (Riddle *et al*. 1981, Vowels and Thomas 1992, Thomas *et al*. 1993, Gottlieb and Ruvkun 1994, Malone and Thomas 1994, Larsen *et al.* 1995); thus, we used a similar approach to identify the possible points of interaction between dauer formation and endogenous RNAi pathways. To perform epistasis analysis, we crossed the *mut-16* (*daf-d*) with *daf-7* (*daf-c*) and *daf-2* (*daf-c*) strains of the TGF-β and insulin-like signaling pathways, respectively, and tested the resulting *mut-16*(*pk710*)*; daf-2*(*e1370*) and *mut-16*(*pk710*)*; daf-7*(*e1372*) double mutants for dauer formation phenotypes in response to

high pheromone conditions. We observed that both double mutant strains exhibited percentages of dauer formation (96.6 ± 3.0% and 99.3 ± 0.4%) significantly greater than *mut-16(pk710)* alone (51.7 ± 6.2%), and similar to the individual *daf-2(e1370)* (97.6 ± 1.7%) and *daf-7(e1372)* (100.0 ± 0%) mutant strains (Fig. 7). Since MUT-16 dependent endo-siRNAs target multiple genes in the TGF-β and insulin signaling pathways (Phillips *et al.* 2012), and the *e1370* allele of *daf-2* is a hypomorph (Gems *et al*. 1998), we cannot conclusively interpret these results without additional experiments. However, these results suggest that MUT-16 is not functioning downstream of DAF-2 and DAF-7 in the dauer formation process. Since DAF-2 insulin receptor and DAF-7 TGF-β function early in the dauer formation decision (Fielenbach and Antebi 2008), this observation is consistent with the possibility that *Mutators* and CSR-1 AGO pathway function in neurons to regulate dauer formation in response to environmental stress.

2.3.3 Functional endogenous RNAi pathways are required in neurons for dauer formation

Neurons, specifically amphid neurons, are important centers for sensing pheromone and for the expression of genes with functions in insulin-like signaling and TGF-β dauer formation pathways (Bargmann and Horvitz 1991). Thus, we hypothesized that CSR-1 AGO and *Mutators* are required in neurons for dauer formation. In order to test our hypothesis, we expressed MUT-16 under a pan-neuronal promoter (*rab-3*) in *mut-16*(*pk710*) background and subjected pan-neuronal rescue worms to the high pheromone dauer formation assay (Fig. 8). As expected, wild-type worms formed significant number of dauers (83.6 ± 2.3%) under high pheromone conditions compared to *mut-16(pk710),* which formed 37.7 ± 4.7% dauers. The two independent transgenic lines carrying the *rab-3*p::*mut-16*::*gfp* transgene exhibited levels of dauer formation

significantly greater than *mut-16*(*pk710*) and similar to wild-type, completely rescuing the *daf-d* phenotype of *mut-16*(*pk710*) (Fig. 8). We also tested *mut-16(pk710)* strains expressing *mut-16*::*gfp* in the intestine under the *ges-1* promoter in order to show that RNAi activity required for dauer formation is tissue specific, *i.e.*, restricted to neurons. These results indicate that expression of *mut-16* in the neurons is sufficient to restore dauer formation in response to pheromone. In addition, this result provides evidence for the role of *Mutators* in somatic RNAi and implicates neurons to be the site of RNAi activity for dauer formation.

We next asked whether small RNAs generated in neurons are acting cell autonomously or spreading to other tissue types to affect dauer formation. *C. elegans* experience systemic RNAi, a process by which dsRNA can spread throughout cells and tissue types to regulate gene expression (Fire *et al.* 1998, Timmons and Fire 1998, Tabara *et al*. 1998, Timmons *et al.* 2001, Winston *et al*. 2002). Although neurons are resistant to systemic RNAi, they can experience autonomous RNAi and can export dsRNA to other tissue types in the worm (Tavernarakis *et al.* 2000, Timmons *et al.* 2001, Kamath *et al.* 2003, Calixto *et al.* 2010, Devanapally *et al.* 2015). Systemic RNAi is dependent upon the dsRNA uptake channel, SID-1, that is expressed throughout the worm in non-neuronal cells (Winston *et al.* 2002; Feinberg and Hunter 2003; Jose *et al.* 2009). Thus, to examine if siRNAs are acting cell autonomously in neurons to regulate dauer formation, we performed dauer formation assays using high pheromone concentrations with two alleles of *sid-1— pk3321* and *qt9*. Interestingly, the null mutant *sid-1(qt9)* strain formed significantly more dauers than wild-type and neither of the mutant alleles exhibited *daf-d* phenotypes, indicating that small RNAs generated in

neurons by *Mutator* proteins are regulating neuronally expressed genes that are essential for dauer formation in unfavorable environments (Fig. 9).

Together, these results indicate that endogenous RNAi pathways are required in neurons for dauer formation in response to high pheromone conditions.

2.3.4 MUT-16 function in individual pheromone-sensing neurons is sufficient for dauer formation in response to pheromone stress

Our results showed that MUT-16 is required in neurons for dauer formation. In order to determine if MUT-16 functions in specific neurons, we generated neuron specific rescues for ADL, ASG, ASJ, ASI, ADF, and AWA neurons and subjected these strains to high pheromone dauer formation assays (Fig. 10). We chose to test ASG, ASJ, ASI, and ADF neurons due to their well-documented roles in dauer formation (Bargmann and Horvitz, Schackwitz *et al*. 1996). We also tested ADL neuron as it has been implicated in promoting dauer formation although having a minor modulatory role (Schackwitz *et al*. 1996, Neal *et al*. 2016). Additionally, ADL neurons have the ability to detect ascr#3 in adult stage (Srinivasan *et al*. 2008, Jang *et al*. 2012). The AWA neuron was used as the negative control as it has not been shown to be important for dauer formation to date. We found that expression of *mut-16::gfp* in ASI and ASJ neurons in the *mut-16(pk710)* strain resulted in significantly greater number of dauers compared to *mut-16(pk710)* without the transgene. We also observed a partial rescue of the *mut-16 daf-d* phenotype for one transgenic line expressing *mut-16::gfp* in ASG neurons, and no rescue for expression of *mut-16::gfp* in ADF and AWA neurons (Fig. 10). Although ASJ, ASI, ADF, and ASG neurons have previously been implicated in dauer formation (Bargmann and Horvitz 1991), these results indicate that expression of MUT-16 in either ASI or ASJ neurons, but not ADF or ASG, is sufficient to rescue the *daf-d* phenotypes of

mut-16 (pk710). Interestingly, we observed that expression of *mut-16::gfp* in ADL neurons also resulted in a significant rescue of the *mut-16 daf-d* phenotype. Hence, our results suggest that MUT-16 functions in subsets of neurons in response to high pheromone. ASI, ASJ, and ADL neurons respond to ascr#2 and ascr#3 pheromone (McGrath *et al.* 2011, Jang *et al.* 2012, Park *et al.* 2012, Neal *et al.* 2015), which are the two most abundant and potent components of crude dauer pheromone (Butcher *et al.* 2007, Butcher *et al.* 2008). Our results are consistent with a model that functional RNAi in just one of these pheromone- sensing neurons is sufficient for detection and/or signaling of high pheromone conditions to result in dauer formation.

2.3.5 *mut-16* mutation does not affect cilium structure

In order to verify that *mut-16* mutants are not dauer deficient due to a neuronal structural defect resulting in the inability to sense pheromone, we examined the ability of *mut-16*(*pk710*) and wild-type worms to uptake a lipophilic dye via their cilia. Worms were dye-filled with DiI that is taken up by amphid neurons ASJ, ADL, ASI, and ASK—neurons implicated in dauer formation (Fig. 11). The dye-filling images show that the amphid neurons appear to be dye-filled normally in *mut-16*(*pk710*)(Panel B) mutants similar to wild-type (Panel A). This result indicates that *mut-16* mutants are not defective in dauer formation due to a detectable developmental defect.

2.3.6 RNAi pathways affect complete dauer morphogenesis in dauer inducing conditions

We found that disruption of RNAi components can increase incidence of incomplete dauer formation. When testing the *daf* phenotypes of RNAi pathway mutants, we found wild-type larvae did not form partial dauers in pheromone conditions and formed very few partial dauers (0.1 ± 0.1%) on water control plates. RNAi mutants

drh-3(ne2453)(2 ± 1.3%) formed significantly more partial dauers, comparable to control *daf-c* mutant *daf-2*(*e1370*)(1.3 ± 1.29%) levels in pheromone conditions (Table 1). *csr-1*(*tm892*); *mut-16*(*pk710*) double mutants also displayed significantly higher number of partial dauers (2.5 ± 1.7%)%)(One way ANOVA using LSD post-hoc test, $p<0.0005$). For the remainder of the mutant strains, we observed low numbers of this phenotype in high pheromone conditions showing a binary response to pheromone in choosing to enter either dauer stage or continuous development, consistent with the reports before indicating pheromone to be the strongest trigger of dauer formation (Golden and Riddle 1982)(Table 1).

Formation of partial dauers on control plates could be augmented by 25 °C, which was the incubation temperature used to carry out high pheromone dauer formation assay. Therefore, appearance of partial dauers in high pheromone conditions although in low numbers, indicates that disruption of RNAi pathway components increases the probability of incomplete dauer formation.

When analyzing the results of epistasis experiment (Fig. 7), wild-type worms did not form partial dauers on either high pheromone or water control plates (Table 2). *mut-16(pk710)* did not exhibit any partial dauers in water plates but showed 4.7 ± 2.1% partial dauers in pheromone plates indicating the possible effect the *mut-16* mutation may have on completion of dauer morphogenesis.

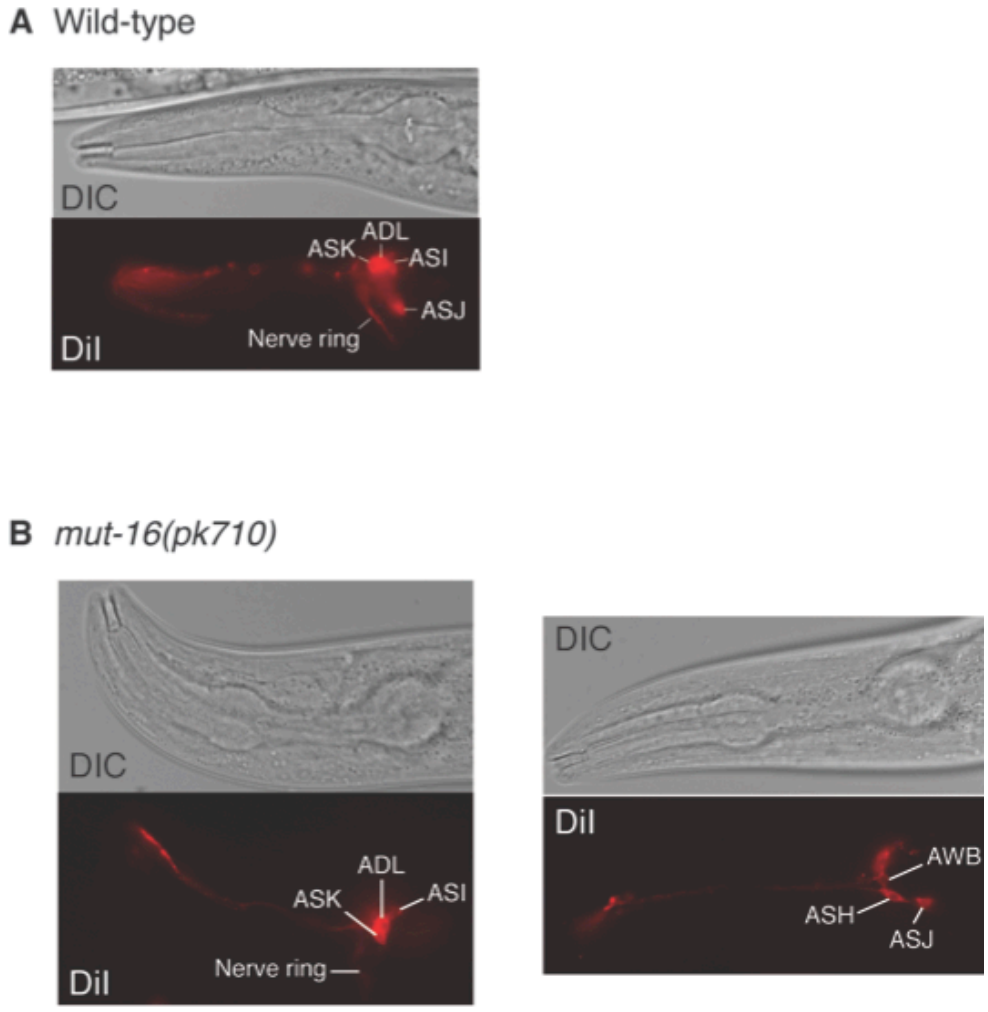


Fig. 11. Dye-filling using DiI stain highlights the cilium structures extending from amphid neurons important for dauer formation (ADL, ASI, ASJ, ASK). The *mut-16*(*pk710*)(panel **B**) do not diplay obvious abnormal cilium phenotype and appear similar to wild-type (panel **A**). This experiment shows that *mut-16* mutants exhibit no ciliary malfunction, which therefore does not contribute to the *daf-d* phenotype of *mut-16*(*pk710*).

TABLE 1: Percentage of partial dauers displayed by RNAi mutants in high pheromone dauer formation assay

Genotype	**Water**	**Pheromone**
Wild-type	0.1	0.0
mut-7(pk720)	0	0.7
mut-8(ne221)	0.1	1.1
mut-16(pk710)	0	0.1
csr-1(tm892)	0	1.5
mut-16(pk710); csr-1(tm892)	0	2.5
drh-3(ne4253)	0	2.1
daf-2(e1370)	3.6	1.2
daf-7(e1372)	0.7	0

The mutants not listed here did not exhibit any partial dauer phenotype.
The phenotypes observed here correspond to results section 2.3.1

Additionally, the *daf-2;mut-16* and *daf-7;mut-16* double mutants exhibited increased partial dauer formation on water plates and fewer partial dauers forming on pheromone plates. Appearance of partial dauers on water plates could be due to the effect of 25°C incubation temperature that induces dauer formation which was perhaps not penetrable enough to successfully complete the dauer morphogenesis.

For the dauer formation assays using tissue-specific *mut-16* rescues (Fig. 8), only intestinal rescue line #1 (9.8 ± 5.9%) formed significantly more number of partial dauers than wild-type (0%) and *mut-16(pk710*)(0.7 ± 0.7%)(Table 3).

TABLE 2: Epistasis analysis- Percentage partial dauers in high pheromone dauer formation assay

Genotype	**Water**	**Pheromone**
Wild-type	0	0
mut-16(*pk710*)	0	4.0
daf-2(*e1370*)	4.7	1.8
mut-16(*pk710*);*daf-2*(*e1370*)	0.7	0
daf-7(*e1372*)	1.2	0
mut-16(*pk710*);*daf-7*(*e1372*)	6.2	0.6

The partial dauer phenotypes observed here correspond to results section 2.3.2

For the neuron specific rescues, we observed varying numbers of partial dauers displayed by rescue lines in both pheromone and water conditions (Table 4). ADL rescue line #2 (9.6± 5.2%) and ASJ rescue line # 1(6.0 ± 2.9%) exhibited significantly more number of partial dauers than wild-type (0.2 ± 0.1%) and *mut-16*(*pk710*) (1.6 ± 0.7%) in high pheromone stress. The transgenic rescues are extrachromosomal lines, which could be the reason for the inconsistent phenotypes between lines for the same rescues.

We also noticed insignificant numbers of partial dauers exhibited by *sid-1* null mutant strain (0.8 ± 0.9%) on pheromone plates but none for wild-type or the *sid-1*(*pk3322*) strains (Table 5). There were no partial dauers on control plates.

Thus, RNAi pathway mutants consistently showed increased number of partial dauers in high pheromone conditions, which may have been facilitated by extrinsic factors such as incubation temperature.

TABLE 3: Percentage partial dauers in high pheromone dauer formation assay for pan neuronal *mut-16* rescue

Genotype	**Water**	**Pheromone**
Wild-type	0.1	0.0
mut-16(pk710)	0	0.7
Pan neuronal rescue 1	0	0.1
Pan neuronal rescue 2	0	2.1
Non neuronal rescue 1	0	9.8
Non neuronal rescue 2	0	3.3

The partial dauer phenotypes observed here correspond to results section 2.3.3

2.3.7 CSR-1 and *Mutators* promote expression of G protein genes required for dauer formation

Our results thus far have shown that MUT-16 expression in the ASI, ADL and ASJ neurons is required for dauer formation. These neurons detect dauer inducing pheromone molecules, thereby mediating dauer entry (McGrath *et al.* 2011, Jang *et al.* 2012, Park *et al.* 2012, Neal *et al.* 2015). ASI is known to detect ascr#2 and ascr#5 ascarosides in larva and ADL is known to detect ascr#3 specifically in adults (Thomas and Robertson, 2008, Kim *et al*. 2009, Park *et al*. 2012, Jang *et al*. 2012). In *C. elegans* neurons, GPCRs and associated proteins localize to the membrane of cilia and serve to detect specific environmental stimuli, including individual pheromone components.

TABLE 4 : Percentage of partial dauer phenotype displayed by *mut-16* neuron specific rescues in high pheromone dauer formation assay

Genotype	Water	Pheromone
Wild-type	0	0.2
mut-16(pk710)	0	1.6
ADL #2	0	9.6
ASG #2	0	0.6
ASJ #1	2.2	6.0
ASJ #2	1.3	2.3
ASI # 1	0	0.5
ASI #2	1.4	3.2
ADF #1	0	0.6
AWA #1	0	0.2

The transgenic lines not listed here did not exhibit any partial dauer phenotype. The phenotypes observed here correspond to results section 2.3.4

Upon ligand binding, GPCRs activate G proteins, which function to transduce the sensory signal intracellularly (Koelle 2016). Based on this information we hypothesized that *Mutator*-amplified siRNAs regulate the expression of genes that play a role in the detection and signaling of high pheromone concentrations via the CSR-1 pathway. In order to understand the potential molecular mechanism by which endogenous RNAi regulates pheromone sensation, we sought to identify candidate genes involved in sensory signaling that are expressed in ASI, ASJ, and ADL neurons. The CSR-1 pathway has been shown to positively regulate the transcription of endogenous genes (Avgousti *et al*. 2012, Conine *et al*. 2013) through interactions with RNA polymerase II (Cecere *et al*. 2014) and maintenance of a euchromatic chromatin state at target gene

loci (Claycomb *et al*. 2009, Seth *et al*. 2013, Wedeles *et al*. 2013). Thus, we hypothesized that gene expression of CSR-1 targets required for dauer formation would be down-regulated in the *mut-16* and *csr-1* hypomorph strains, resulting in their observed *daf-d* phenotypes. We identified three genes encoding G protein subunits, *gpa-1* (G protein α), *gpa-3* (G protein α), and *gpc-1* (G protein γ), that meet these criteria and exhibit *daf-d* phenotypes in their respective loss-of-function mutant strains in response to high pheromone concentrations (Zwaal *et al.* 1997, Lans and Jansen 2007, Kim *et al.* 2009).

TABLE 5 : Percentage partial dauers in high pheromone dauer formation assay for *sid-1* mutants

Genotype	**Water**	**Pheromone**
Wild-type	0	0
sid-1(qt9)	0	0.8

The mutants not listed here did not exhibit any partial dauer phenotype
The partial dauer phenotypes observed here correspond to results section 2.3.3

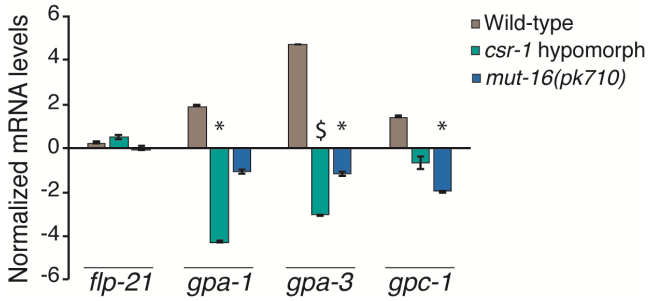


Fig. 12. *Mutator* MUT-16 and CSR-1 AGO promote expression of chemosensation genes required for dauer formation. Log2 mRNA levels for *flp-21*, *gpa-1*, *gpa-3*, and *gpc-1* genes in wild-type, *csr-1* hypomorph, and *mut-16(pk710)* strains, normalized to *y45f10d.4*, are shown. * $p < 0.05$ and \$ $p < 0.005$ compared to wild-type, one-way ANOVA with LSD post hoc test. N ≥ 3 biological replicates. Error bars represent propagated standard deviation.

To examine if *Mutator* proteins and CSR-1 are playing a role in the regulation of these genes, we measured their mRNA levels in larval L1 stage animals in wild-type, *csr-1* hypomorph, and *mut-16(pk710)* strains. We found that mRNA levels of *gpa-1* and *gpa-3* were significantly reduced in the *csr-1* hypomorph indicating that the CSR-1 pathway promotes expression of these G proteins during the L1 larval stage. Also, *gpa-3* and *gpc-1* were significantly downregulated in *mut-16(pk710)* background compared to wild-type (Fig. 12). In order to verify that the expression pattern of *gpa-1*, *gpa-3*, and *gpc-1* is specific to the mutant backgrounds, we also checked the expression of *flp-21*, another gene expressed in ADL, ASJ, and ASI neurons. Mutation in *flp-21* has not been shown to exhibit any dauer formation defects in response to high pheromone concentration. We found the expression of *flp-21* to be the same across all genotypes (Fig. 12). These results are consistent with our hypothesis that the *daf-d* phenotypes exhibited by the *csr-1* hypomorph and *mut-16(pk710)* strains in response to high pheromone conditions are a result of decreased G protein signaling in neurons that allow detection of high concentrations of ascaroside molecules. Together, our results indicate that endogenous RNAi pathways are required in distinct subsets of neurons for appropriate dauer formation in response to different environmental stresses, and provide evidence that the CSR-1 pathway promotes expression of genes essential for sensory signaling early in *C. elegans* development.

2.4 Conclusion

In this chapter, we provide evidence that endogenous RNAi pathways are required for the dauer formation decision in *C. elegans*. We showed that strains carrying mutations in *Mutator* proteins and the nuclear CSR-1 AGO pathway exhibit *daf-d*

phenotypes in response to high pheromone conditions. In addition, we demonstrate that MUT-16 function is required in distinct subsets of neurons for dauer formation in response to stress. Furthermore, our data indicates that CSR-1 promotes expression of genes essential for sensory signaling in neurons that have been shown to detect individual ascarosides.

Chapter 3

RNAi is required in high temperature stress for dauer formation

3.1 Synopsis

Dauer formation can also be induced by high temperature (27°C), although the neural and molecular mechanisms underlying this behavior are yet to be fully understood (Golden and Riddle, 1984a, b, Hobert *et al*. 1997, Ailion and Thomas 2000, Ailion and Thomas 2003). As described in Chapter 2, we showed that mutations in RNAi pathway genes affect the ability of *C. elegans* to form dauers in response to pheromone stress. Next, we sought to verify if this *daf* phenotype persists in elevated temperature conditions. Upon conducting high temperature dauer formation assays, we verified that CSR-1 and a distinct subset of *Mutator* proteins are required for dauer formation in high temperatures, indicating that different stresses have distinct requirements for dauer formation. Similar to high pheromone conditions, RNAi is required in neurons for dauer formation at elevated temperature; however, the specific neurons where RNAi is required for dauer formation in response to high temperature are distinct from those required in high pheromone. Identification of specific neurons or subsets of neurons where RNAi pathway function is required for high temperature induced dauer formation will further our understanding of how temperature is integrated in the neural circuit for dauer developmental decisions.

3.2 Methods

3.2.1 Cloning and generation of *mut-16* rescue strains

The method followed to generate transgenic *mut-16* pan-neuronal and neuron specific rescue lines (listed in Appendix 1) is described in Chapter 2 (section 2.2.1).

3.2.2 High temperature dauer formation assays for scoring *daf* phenotypes at elevated temperatures

In order to characterize the *daf* phenotypes of RNAi pathway mutants in response to high temperature, we developed a dauer formation temperature assay that was modified from previous published protocols (Ailion and Thomas 2000, Ailion and Thomas 2003). These protocols required optimization due to the fact that RNAi pathway mutants showed severe sterility phenotypes and sensitivity to higher temperatures. As a result, we modified the timing of when the progeny were moved to high temperatures after hatching (L1 larvae). In the original protocol, embryos were allowed to hatch at high temperatures. Our modified protocol is described in further detail below.

Our high temperature dauer formation assay involves subjecting newly hatched worms to high temperatures in order to induce dauer formation. The preparation of assay plates was carried out in a similar manner as described in section 2.2.1 with the omission of pheromone in assay plates. The egg laying procedure was also carried out similar to the pheromone dauer formation assay with the modification that we waited 10-12 hours for the eggs to hatch at room temperature before shifting the plates to the incubators (25°C or 27°C) to prevent embryonic lethality or L1 diapause. Incubation temperatures of 25°C and 27°C are known to induce intermediate and high levels of dauer formation in wild-type, respectively (Ailion and Thomas 2000). At all temperatures, a wet paper towel was placed in the incubator boxes to prevent the plates

from desiccation and was replaced everyday. Assay plates were scored for the presence of dauers after 4 days. The dauers were scored by checking for the complete absence of the pharynx pumping activity and the appearance of the alae (Cassada and Russell, 1975, Albert and Riddle, 1988, Popham and Webster 1979, Riddle and Albert 1997). Temperature stress can result in increased number of partial dauers in certain mutant strains; thus, careful examination of the pharynx activity and body structure using a dissection microscope was required. It should also be noted that there is overall fewer dauers induced through high temperature compared to high pheromone stress for wild-type animals.

Each strain was tested independently at least 3 times on separate days. Statistical significance was determined using an ANOVA with LSD post hoc test available in the SPSS software (version 23).

3.3 Results

3.3.1 *Mutators* exhibit stress-specific *daf-d* phenotypes

We questioned if the disruption of *Mutator* and CSR-1 pathway proteins also exhibited *daf-d* phenotypes in response to different environmental stresses. First, we subjected newly hatched L1 worms to elevated temperatures (25°C and 27°C) and measured the proportion of dauer formation (Fig. 13). At 27°C wild-type formed dauers (45.6 ± 2.6%) that were characterized by the appearance of prominent alae, thickening of the cuticle, and lack of pharyngeal activity (Cassada and Russell, 1975, Albert and Riddle, 1988, Popham and Webster 1979, Riddle and Albert 1997). *daf-2(e1370*) and *daf-7*(*e1372*) controls exhibited *daf-c* phenotype as expected at all temperatures (Riddle *et* al. 1981, Vowels and Thomas 1992, Gottlieb and Ruvkun 1994). We also noted that

the *daf-3*(*mgDf90*) strain exhibited a *daf-d* phenotype at 25°C (5.4 ± 3.8%), but a *daf-c* phenotype at 27°C (71.4 ± 13.0%), consistent with previous reports (Ailion and Thomas, 2000). Compared to wild-type, *mut-2*(*ne298*) (29.6 ± 8.6%), *mut-15*(*tm1358*) (13.7 ± 9.4%), *mut-16*(*pk710*) (22.5 ± 4.0%), *rrf-1*(*pk1417*) (20.6 ± 4.9%) and *csr-1* hypomorph (11.0 ± 3.3%) strains formed significantly fewer dauers at 27°C (Fig. 13). Interestingly, except for *mut-16*(*pk710*) and *csr-1* hypomorph, these mutant strains formed dauers at moderately high temperature (25°C) similar to wild-type (Fig. 13). We were unable to test *drh-3*(*ne2453*) due to its sterility and arrested growth phenotype at this temperature. Moreover, *mut-8*(*ne221*) (23.5 ±13.6%) formed more dauers compared to wild-type at 25°C (9.9 ± 2%). When compared with pheromone dauer formation assay results, we observed that a different subset of *Mutator* genes is required for dauer formation in response to temperature stress, indicating that the RNAi components required for dauer formation are stress-specific.

We also observed that wild-type strains and most of the RNAi mutants showed increased incidence of partial dauers at 27°C compared to that scored at 25°C (Table 6). Overall the high temperature dauer formation assays resulted in higher percentages of partial dauers compared to seen that in high pheromone dauer formation assays (Tables 1-5).

Fig. 13

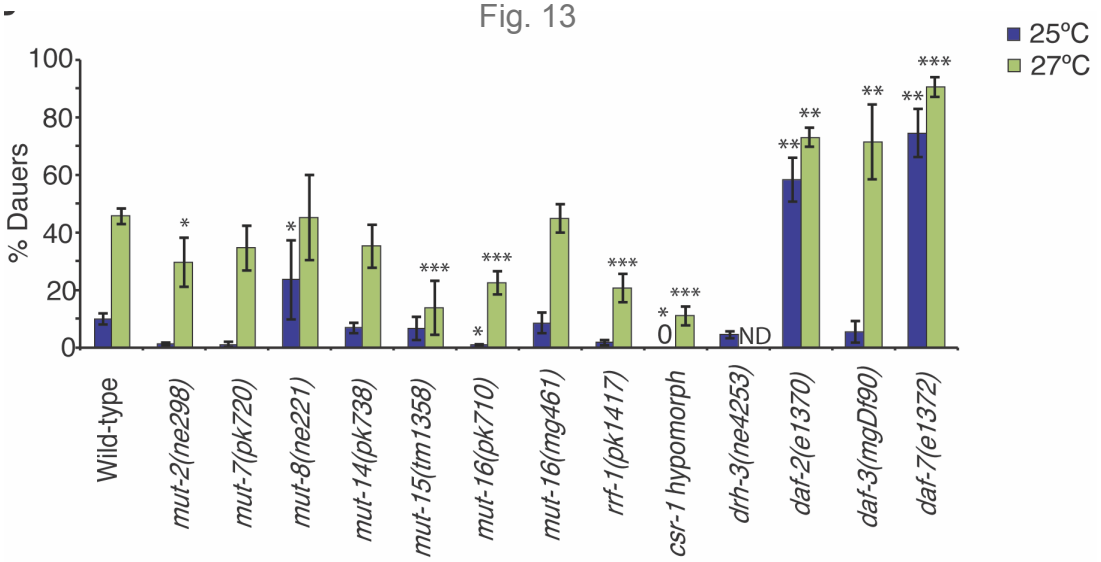


Fig. 14

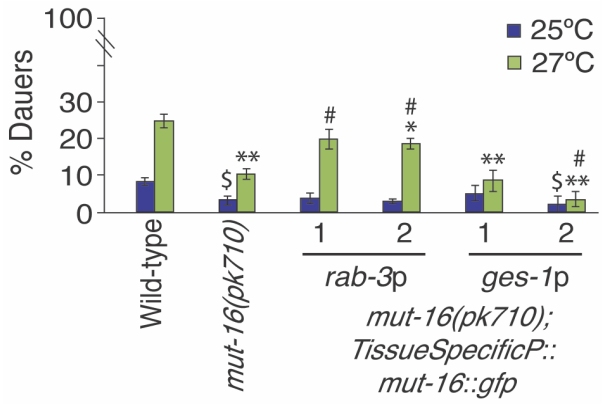


Fig. 13. Proportion of animals forming dauers when cultivated at 25°C and 27°C. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to wild-type, one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 164 animals. **Fig. 14.** Proportion of animals forming dauers in response to 25°C and 27°C is shown for pan-neuronal and intestinal *mut-16* rescue strains. * $p < 0.05$ and ** $p < 0.0005$ compared to wild-type at 27°C; # $p < 0.005$ compared to *mut-16(pk710)* at 27°C; $ $p < 0.05$ compared to wild-type at 25°C; one-way ANOVA with LSD post hoc test. N = 3 trials; n ≥ 119 animals. All error bars represent S.E.M.

TABLE 6: Percentage of RNAi mutants exhibiting partial dauer phenotype at high temperatures

Genotype	**25°C**	**27°C**
Wild-type	0.2	12.6
mut-2(ne298)	0.2	30.0
mut-7(*pk720*)	0	9.6
mut-8(*ne221*)	0.3	0
mut-14(*pk738*)	3.9	38.3
mut-15(*tm1358*)	0.9	0
mut-16(*pk710*)	0.1	26.9
mut-16(*mg461*)	0	33.0
rrf-1(*pk1417*)	0	0.7
csr-1 hypomorph	0.7	17.3
drh-3(*ne4253*)	1.3	0
daf-2(*e1370*)	23.2	13.5
daf-3(*mgDf90*)	0	12.0
daf-7(*e1372*)	1.6	2.9

The mutants not listed here did not exhibit any partial dauer phenotype.
The phenotypes observed here correspond to results section 3.3.1

3.3.2 RNAi activity in neurons is required for dauer formation at high temperatures

Since our model predicts that endo-RNAi pathways are regulating genes in neurons important for sensory detection and/or signaling of dauer-inducing high

pheromone conditions, we hypothesized that MUT-16 would be required to regulate dauer formation in response to other stressors including elevated temperature. To

Fig. 15

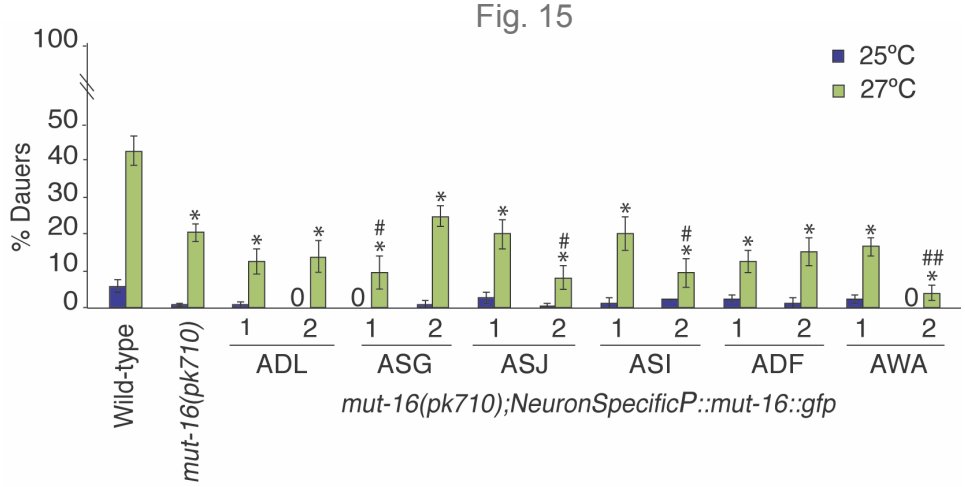


Fig. 15. Proportion of animals forming dauers in response to 25°C and 27°C is shown for neuron-specific *mut-16* rescue strains. * represents $p < 0.0005$ compared to wild-type at 27°C; # $p < 0.005$ and ## $p < 0.0005$ compared to *mut-16*(*pk710*) at 27°C; one-way ANOVA with LSD postdoc test. N ≥ 3; n ≥ 120 at 27°C and N ≥ 3; n ≥ 146 at 25°C. All error bars represent S.E.M.

address our hypothesis, we first subjected two *mut-16::gfp* pan-neuronal rescues to high temperature dauer formation assays (Fig. 14). Both the transgenic lines expressing *mut-16* under a pan-neuronal promoter (*rab-3*) exhibited increased number of dauers (19.8 ± 2.6% and 18.7±1.4% for lines #1 and #2, respectively) compared to *mut-16*(*pk710*) (11.8 ± 0.8%) at 27°C (Fig. 14). The *daf-d* phenotype of *mut-16* (*pk710*) was also rescued at 25°C shown by increased number of dauers in the rescue lines not significantly different from wild-type phenotype (Fig. 14). On the other hand, transgenic lines harboring *mut-16* driven by intestine promoter (*ges-1*) exhibited *daf-d* phenotype similar to *mut-16*(*pk710*) levels (8.5 ± 3.0% and 3.5 ± 1.9% for lines #1 and #2, respectively) (Fig. 14).

It is also worth noting there is increased number of partial dauers exhibited by the rescue lines at 25°C and 27°C (Table 7). Given that the rescues are extrachromosomal lines, this is not surprising since the mutants were expressing more than one copy of *mut-16*, generating genetic mosaics. Neither the wild-type or the *mut-16*(*pk710*) strains formed any partial dauers.

TABLE 7: Percentage of *mut-16* tissue specific rescues displaying partial dauers in high temperature dauer formation assay

Genotype	**25°C**	**27°C**
Wild-type	0	0
mut-16(pk710)	0	0
Pan neuronal rescue 1	1.2	18.3
Pan neuronal rescue 2	5.6	0
Non neuronal rescue 1	6.6	6.5
Non neuronal rescue 2	2.4	2.7

The mutants not listed here did not exhibit any partial dauer phenotype.
The phenotypes observed here correspond to results section 3.3.2

Overall, these results indicate that endogenous RNAi pathways are required in neurons for dauer formation when subjected to at least two types of environmental stresses — temperature and pheromone. We next tested whether the neuron-specific MUT-16 rescue strains exhibited increased dauer formation in response to high temperature. At 27°C, all of the neuron specific rescues tested formed percentage dauers that were not significantly different from *mut-16*(*pk710*) levels. These results indicate that MUT-16 is not sufficient in ASJ, ASI, ADF, ASG, ADL, or AWA alone to

regulate dauer formation in response to high temperature but may be required in single neurons that remain untested or in a subset of neurons (Discussed in detail in section 6.3)(Fig. 15). Overall, 27°C for certain rescue lines induced a larger number of partial dauers compared to 25°C. Neuron specific lines ADL #2 (20.2 ± 12.8%), ASG #2 (47.7 ± 3.0%), ASJ #1(32.5 ± 10.1%), ADF #1 (36.4 ± 5.9%), and ADF #2 (25.9 ± 9.6%) formed significantly large numbers of partial dauers compared to wild-type (7.2 ± 2.8%) and *mut-16*(*pk710*)(15.7 ± 5.6%)(Table 8). At 25°C, these strains did not form any partial dauers.

TABLE 8: Percentage of *mut-16* neuron specific rescues displaying partial dauer phenotype in high temperature dauer formation assay

Genotype	**25°C**	**27°C**
Wild-type	0.1	7.2
mut-16(pk710)	0	15.7
ADL #1	0	0.4
ADL #2	0	20.2
ASG #1	0	13.5
ASG #2	0	47.7
ASJ # 1	0	32.5
ASJ #2	1.1	14.6
ASI #1	0	3.6
ASI #2	0.3	7.3
ADF #1	0	36.4
ADF #2	0	25.9

The transgenic lines not listed here did not exhibit any partial dauer phenotype.
The phenotypes observed here correspond to results section 3.3.2

Together, these findings give us insight on how endogenous RNAi pathways are required for dauer formation, and are consistent with a model that the *Mutators* and CSR-1 pathways act in specific subsets of neurons required for dauer formation in response to different environmental cues.

3.4 Conclusion

In this chapter we show that RNAi pathways are also required for dauer formation in high temperatures. Given that our results show that distinct subsets of *Mutators* are required for dauer formation in response to pheromone or high temperature, we speculate that the *Mutator* complex may not necessarily operate as a unit in neurons as is does in the germ line and that the requirement of individual components may be contingent upon the nature of stress experienced. Although we have shown that MUT-16 is required in neurons for high temperature dauer formation, the precise location of MUT-16 function is yet to be determined.

Chapter 4

RNAi is required for dauer formation in starvation conditions

4.1 Synopsis

Availability of food is a limiting factor for *C. elegans* growth and reproduction, and the lack of it can create unfavorable conditions leading to dauer formation. After testing whether components of the RNAi pathway regulate pheromone and temperature induced dauer formation, the next step was to investigate if mutations in *Mutator* and CSR-1 pathway genes exhibited *daf* phenotypes in starvation conditions (Chapter 2 and Chapter 3). To achieve this goal, we designed specialized starvation assays to accommodate for the reduced fecundity of the strains tested. The results of dauer formation assays using starvation showed that a different subset of *Mutators* were required for starvation-induced dauer formation compared to high pheromone conditions. Similar to the results of high temperature dauer formation assays, we show that MUT-16 is required in neurons for dauer formation; however, expression of MUT-16 in the individual neurons tested did not rescue the *mut-16*(*pk710*) *daf-d* phenotype. Since the neuronal and molecular mechanisms regulating dauer formation in response to starvation stress are not as well characterized as for high pheromone conditions (Hu 2007), this study has the potential to uncover the neurons where dauer RNAi is required for dauer formation in the starvation condition.

4.2 Methods

4.2.1 Cloning and generation of rescue strains

The method followed to generate transgenic *mut-16* pan-neuronal and neuron specific rescue lines (listed in Appendix 1) is described in Chapter 2 (section 2.2.1).

4.2.2 Starvation-induced dauer formation assay

The starvation-induced dauer formation assay is based on the understanding that worms assess the 'food to population ratio' in their environment (Golden and Riddle 1984). The protocol that was commonly followed to characterize *daf* phenotypes through starvation assay was semi-quantitative that did not control the number of worms present and the amount of food available to them before starving them for entry into dauer stage (Malone and Thomas 1996).

For our purposes, we developed specialized assays that carefully controlled the number of animals and amount of food present on the dauer formation assay plates. First, we optimized the minimum amount of food required for the worms to enter dauer stage. The plate preparation was the same as that used in the high temperature dauer formation assay (Chapter 3, section 3.2.2). Eight wild-type young adults were allowed to lay eggs for approximately 12 hours on plates with varying amounts of heat killed *E. coli* (OP50) (0.024 mg, 0.028 mg, 0.036 mg, 0.04 mg, 0.16 mg) at room temperature (22°C). The adults were removed and the plates were shifted to 25°C incubator for 5 days. The assay plates containing 0.04 mg of heat killed OP50 generated maximum number of dauers (Fig. 16). Plates seeded with lesser amounts of food (0.024 mg and 0.028 mg) resulted in increased numbers of larvae entering L1 arrest or L1 diapause stage. This is consistent with the previous reports that describe increased incidence of L1 arrest in the

absence of food and that presence of limited amount of food is required for worms to enter dauer, although not conducive enough for reproduction and population growth (Baugh 2013, Baugh and Sterberg 2006). Accordingly, the *daf* phenotype of RNAi mutants in starvation conditions was determined by allowing adults to lay eggs that hatched in the presence of 0.04 mg of heat killed OP50. Some of the mutant strains tested (*csr-1* hypomorph, *mut-16*(*pk710*), *mut-2*(*ne298*), *mut-7*(*pk720*), and *drh-3*(*ne2453*) exhibited significantly reduced brood sizes. One of our challenges was to obtain similar number of progeny on all assay plates across genotypes. The mutant adults do not make the same number of progeny as wild-type and adding extra adults meant depletion of food source. Additionally, allowing the mutant adults to continue laying eggs for extended amounts of time could result in excess depletion of food present on the plates. Thus, in order to compensate for the partial sterility of these mutant strains and to accumulate required number of progeny on assay plates, we transferred 2 transgenic wild-type worms expressing *unc122*p*::dsRed* marker (SH48, Appendix 1) in addition to 6 mutant adults. After allowing the worms to lay eggs for 12 hours, the adults were removed and embryos were moved to the 25°C incubator for 5 days. At the time of scoring, the mutant and wild-type worms were distinguished by the presence of dsRed; the *daf* phenotypes of the mutants alone were reported. The characteristics typically displayed by dauers, *i.e*., no pharynx activity and appearance of alae was used to distinguish dauers from non-dauers, as described for other assays in Chapters 2 and 3.

Starvation induced dauer formation assays were performed at least 3 times with biologically independent samples of each strain (Appendix 1). Statistical significance of

data was determined with a One-way ANOVA with a LSD post hoc correction using SPSS software (version 23).

4.3 Results

4.3.1 A Subset of *Mutators* and the CSR-1 AGO pathway are required for dauer formation in starvation conditions

We measured dauer formation in response to starvation conditions by subjecting newly hatched worms to depleted amounts of *E. coli* (Fig. 17). Control strain *daf-7*(*e1372*) formed a significant number of dauers as anticipated (89.6 ± 0.6%) and *daf-3*(*mgDf90*) (1.9 ± 1.2%) formed significantly less dauers resembling the *daf-d* mutants. We observed that *mut-2*(*ne298*) (3.6 ± 0.9%), *mut-7*(*pk720*) (1.5 ± 0.5%), *mut-14*(*pk738*) (2.7 ± 1.6%), *mut-15*(*tm1358*) (4.9 ± 1.2%), and *mut-16*(*mg461*) (10.1 ± 4.1%), *mut-16* (*pk710*) (8.2 ± 1.8%), and *csr-1* hypomorph (3.0 ± 1.2%) strains exhibited significant *daf-d* phenotypes compared to wild-type (19.9 ± 2.3%) when exposed to starvation conditions early in development. However, *mut-8*(*ne221*) (14.8 ± 3.5%) and *rrf-1*(*pk1417*) (25.4 ± 3.3%) formed dauers not significantly different from wild-type. From these results, we concluded that a different subset of *Mutators* than that seen in pheromone and temperature stress are required in starvation conditions for regulating dauer formation.

Overall, our results suggest that MUT-2, MUT-7, MUT-16, MUT-14, MUT-15, and CSR-1 are required to regulate dauer formation in starvation conditions. It should be noted that in our assays, wild-type animals formed fewer dauers in response to starvation as compared to both high pheromone and elevated temperature. We also found both *mut-16* alleles exhibiting partial dauer phenotypes (Table 9). *mut-16*(*pk710*) and *mut-16*(*mg461*) exhibited 9.3 ± 4.9% and 7.04 ± 3.7% partial dauers, respectively.

Wild-type did not form any starvation-induced partial dauers implying that disruption of RNAi can increase the incidence of partial dauers in starvation stress (Table 9). It is possible that disruption in the RNAi pathway may cause errors in complete transition into dauer morphology.

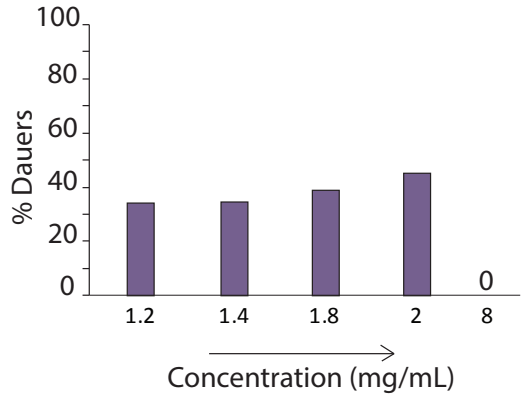


Fig. 16. Optimization of amount of heat-killed OP50 *E. coli* required to induce dauer formation in starvation conditions. Maximum number of dauers was induced at 2mg/mL of heat-killed bacteria. 8 mg/mL was used as the control.

TABLE 9: Percentage of partial dauer phenotype displayed by *Mutator* mutants in starvation condition

Genotype	**2mg/ml OP50**
Wild-type	0
mut-16(*pk710*)	9.3
mut-16(*mg461*)	7.0

The transgenic lines not listed here did not exhibit any partial dauer phenotype.
The partial dauer phenotypes observed here correspond to results section 4.3.1

4.3.2 RNAi pathway is required in neurons for dauer formation in starvation conditions

Next, we subjected the *mut-16*(*pk710*) strain carrying pan-neuronal rescue construct *rab-3*p::*mut-16*::*gfp* to starvation-induced dauer formation assays to determine if MUT-16 activity in neurons is required for dauer formation. The expression of the *mut-16*::*gfp* transgene in neurons completely rescued the *daf-d* phenotype of *mut-16*(*pk710*). The pan-neuronal *mut-16* rescue line #1 and line #2 formed 29.4 ± 7.0% and 23.2 ± 3.5% of dauers, respectively, with line #1 exhibiting a significantly higher number of dauers than wild-type (18.2 ± 2.2%) (Fig.18). The intestine specific rescue lines #1 (4.5 ± 2.5%) and #2 (5.7 ± 2.2%) formed fewer dauers similar to *mut-16*(*pk710*) (7.4 ± 1.1%). We did not observe formation of partial dauers in this experiment except for negligible numbers *mut-16*(*pk710*)(0.13 ± 0.1%) and intestine specific rescue line #1 (0.62 ± 0.6%) plates (Table 10). However, we did not observe any rescue of *mut-16*(*pk710*) *daf-d* phenotypes in response to starvation for the neuron-specific MUT-16 expression strains (Fig. 19). The *mut-16*(*pk710*) (7.1 ± 0.6%) consistently formed significantly fewer dauers than wild-type (24.4 ± 1.7%). MUT-16 activity in the individual neurons ADL, ASG, ASJ, ASI, ADF and AWA resulted in a small percentage of dauers similar to *mut-16*(*pk710*). We noticed a significant decrease in dauer formation in one line of ASI-specific MUT-16 expression. This is interesting given the known role of ASI in integration of food signals in starvation conditions that ultimately facilitate dauer formation (Neal *et al*. 2015)(Chapter 1, section 1.4.4).

Fig. 17

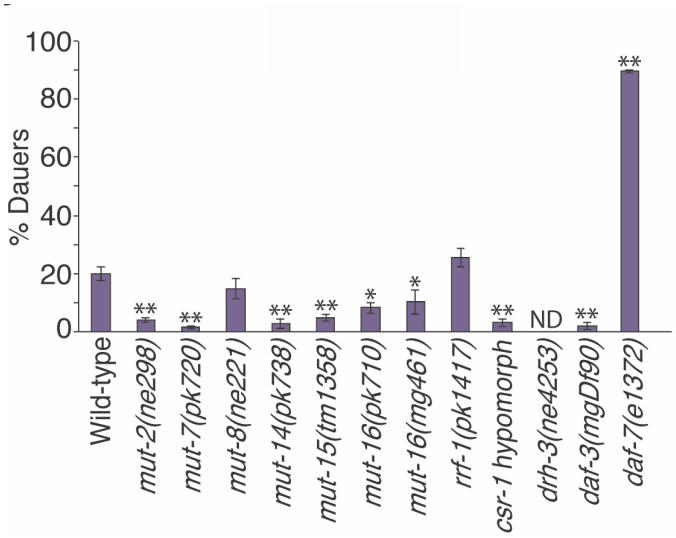


Fig. 18

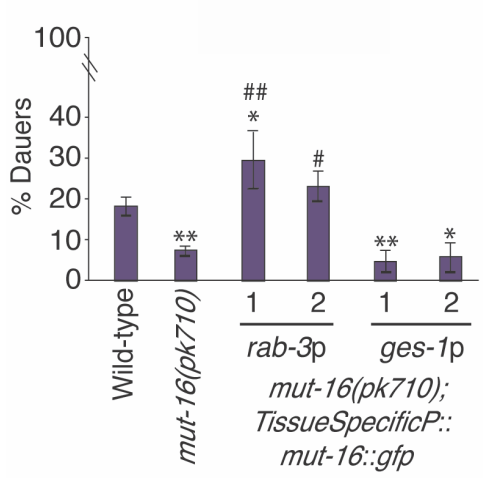


Fig. 19

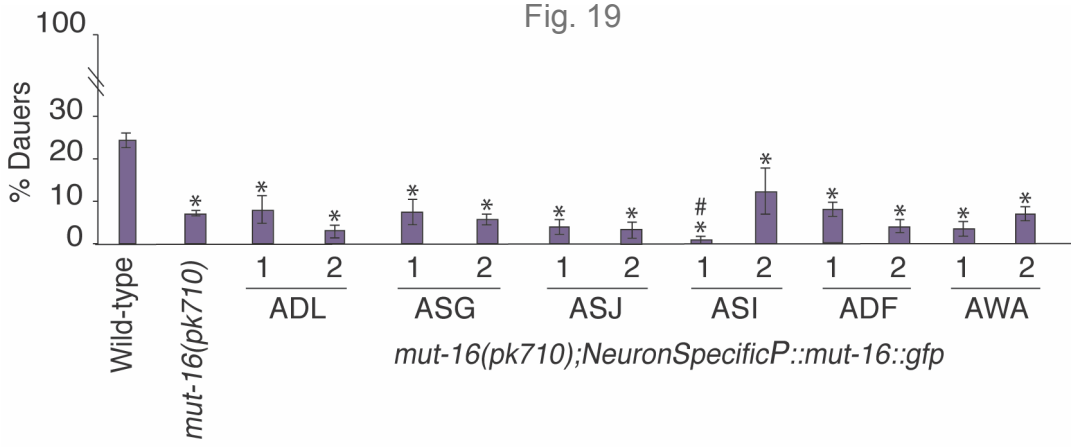


Fig. 17. Proportion of animals forming dauers when subjected to starvation condition. * $p <$ 0.005, ** $p < 0.0005$ compared to wild-type, one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 300 animals. "ND" indicates not determined; "0" indicates no dauers were formed. All error bars represent the standard error of the mean, S.E.M. **Fig. 18**. Proportion of animals forming dauers in response to starvation conditions for pan-neuronal and intestinal *mut-16* rescue strains. * $p < 0.05$ and ** $p < 0.005$ compared to wild-type; # $p < 0.005$ and ## $p < 0.0005$ compared to *mut-16*(*pk710*); one-way ANOVA with LSD post hoc test. N = 3 trials; n ≥ 196 animals. **Fig. 19.** Proportion of animals forming dauers in response to starvation conditions for neuron-specific *mut-16* rescue strains. * $p < 0.0005$ compared to wild-type; # $p < 0.05$ compared to *mut-16*(*pk710*) using one-way ANOVA with LSD post hoc test. N ≥ 3; n ≥ 160. No dauer formation is indicated by "0". Two independent transgenic lines for each neuron-specific rescue were tested. All error bars represent S.E.M.

We know expression of *cmk-1* encoding the CaMKI enzyme is required in AWC neuron (in coordination with ASJ) to regulate ILP synthesis as a function of feeding state. In parallel, *cmk-1* expression in ASI is needed to regulate *daf-7* TGF-β gene. Together these pathways are required for signaling information regarding lack of food availability in the process of dauer formation (Neal *et al*. 2015). Therefore, reduced number of dauers in ASI rescue line #1 could be because expressing *mut-16* in ASI alone may not be sufficient to rescue the *daf* phenotype. Perhaps expressing MUT-16 in subsets of neurons would increase the number of dauers to wild-type levels. It is possible that certain neurons are indispensable for the additive, synergistic or antagonistic interactions leading to dauer formation.

In this experiment, we observed almost no partial dauer formation except for the ASJ neuron rescue line #1 (1.6 ± 1.7%) demonstrating an increased ability to enter dauer stage by the neuron specific rescue strains tested in the starvation condition (Table 11).

4.4 Conclusion

Our results suggest that CSR-1 and the *Mutators* modulate appropriate development decision in starvation stress conditions. We have not yet identified the specific neurons where MUT-16 is required for dauer formation during exposure to this stress; however, our model predicts that it would be in the neurons that detect low food availability. Although the chemical identity of the 'food signal' is yet to be identified, the presence of food (*E. coli*) is integrated by CAMKII in ASI and non-cell autonomously in AWC (Neal *et al*. 2015). However, unlike in high pheromone dauer formation assays,

TABLE 10: Percentage of partial dauer phenotype displayed by *mut-16* tissue specific rescues in starvation condition

Genotype	**2 mg/ml OP50**
Wild-type	0
mut-16(pk710)	0.1
Pan neuronal rescue 1	0
Pan neuronal rescue 2	0
Non neuronal rescue 1	0.6
Non neuronal rescue 2	0

The transgenic lines not listed here did not exhibit any partial dauer phenotype.
The partial dauer phenotypes observed here correspond to results section 4.3.2

TABLE 11: Percentage of partial dauer phenotype displayed by *mut-16* neuron specific rescues in starvation condition

Genotype	**2mg/ml OP50**
Wild-type	0
mut-16(*pk710*)	0
ASJ #1	1.6

The transgenic lines not listed here did not exhibit any partial dauer phenotype.
The partial dauer phenotypes observed here correspond to results section 4.3.2

we have shown that MUT-16 expression in ASI neurons is not sufficient to rescue the *daf-d* phenotype of *mut-16(pk710)* in starvation conditions. More experiments are needed to identify specific neurons for RNAi activity and we speculate such mechanisms may control sensation of absence of food. Overall our results indicated

that subsets of *Mutators* are important for starvation induced dauer formation and are different from that seen in response to other conditions. This might be an implication of how different stressors use distinct subsets of genes and possibly distinct neurons for dauer formation.

Chapter 5

Role of RNAi pathways in regulation of dauer recovery in *C. elegans*

5.1 Synopsis

We successfully developed dauer formation assays to characterize *daf-d* phenotypes of strains carrying mutations in *Mutators* and CSR-1 pathway components in response to distinct environmental stresses. During experiments characterizing *mut-7*(*pk720*) phenotype in dauer formation assays, we observed that *mut-7*(*pk720*) exhibited a second phenotype: delayed recovery of dauers to larval L4 stage compared to wild-type, despite being subjected to favorable conditions. MUT-7 has been shown to be important for promoting odor adaptation in AWC neuron (Juang *et al*. 2013). Additionally, mutation in *mut-7* can result in olfactory defects in response to certain odorants in adults (A. D. Hager and S. E. Hall, unpublished data). Here we propose a novel function for MUT-7 in dauer recovery, which is also likely to be regulated in neurons. We hypothesize that MUT-7 functions in ASJ for dauer recovery given its role in dauer exit (Bargmann and Horvitz 1991).

Previous studies found that presence of food is necessary for dauer recovery even though the identity of the food signals is yet to be fully understood. Keeping this requirement in mind we developed assays that defined the inability of worms to recover from dauer stage, despite the prevalence of favorable conditions, as "*daf-r*" phenotype. Hence, dauer recovery assay helped us test the ability of worms to exit dauer stage on exposure to favorable conditions (availability of food and low population density).

The results of dauer recovery assay indicated that MUT-7 is indeed important for dauer recovery. Therefore, our result suggests an additional role for MUT-7 in larval developmental decisions.

5. 2 Methods

5.2.1 Dauer recovery assay

We developed a new assay to test the ability of RNAi pathway mutants to exit dauer stage. First, the pheromone-induced dauer formation was conducted as described in Chapter 2. The dauers were identified based on the absence of pharynx activity and appearance of alae (Cassada and Russell 1975, Popham and Webster 1979, Albert and Riddle, 1988, Riddle and Albert 1997). Ten pheromone-induced dauers (48 hours in dauer) for each of the strains were rescued by picking them onto separate NGM plates with live OP50 *E. coli* and incubated at 20°C. The dauer recovery phenotypes were scored after 24 hours. The recovered worms had resumed development to mid-L4 stage and were distinguishable from dauers based on their thickened cuticle and lack of pharyngeal pumping (Cassada and Russell, 1975, Albert and Riddle, 1988, Popham and Webster 1979, Riddle and Albert 1997).

The assays were carried out three times with biologically independent samples for wild-type and *mut-7*(*pk720*). A two-tailed T-test was used to determine the significance of the data (Microsoft Excel 2011).

5.3 Results

Dauer recovery assay results indicated that there were significantly higher number of dauers for *mut-7*(*pk720*) mutants compared to wild-type, 24 hours after exposure to favorable conditions (20°C) (Fig. 20). We observed that 94.3 ± 2.2% of the

wild-type worms exited dauer stage and transitioned into the mid L4 stage. Only 37.5 ± 13.2% of *mut-7* (*pk710*) on the other hand were L4s after 24 hours. Allowing the assay plates to incubate at 15°C during recovery slowed dauer exit, but not to a significant extent; p=0.21 for wild-type$^{15°C}$ versus wild-type$^{20°C}$ and p=0.29 for *mut-7*(*pk720*) $^{15°C}$ versus *mut-7*(*pk720*) $^{20°C}$.

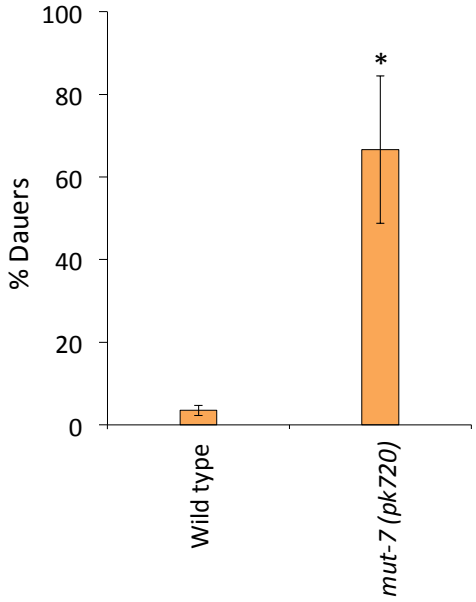


Fig. 20. *mut-7*(*pk720*) is *daf-r* after passage through pheromone induced dauer formation. The graph represents the proportion of L4 animals at 20°C after 24 hours of rescue from pheromone plates. *mut-7* mutant exhibits *daf-r* phenotype. Fewer mutants exited dauer phenotype even after 24 hours of exposure to favorable conditions. * Represents p-value < 0.05 using two-tailed T-test. Error bars represent SEM. Minimum of 300 animals were used to test the dauer recovery phenotype.

5.4 Conclusion

Results from the recovery assay indicate that MUT-7 is required for dauer recovery in *C. elegans* in addition to the dauer entry decision (Chapter 2, 3 and 4). Decrease in population density and access to increased amounts of food are key determinants of dauer recovery for transition of dauers into reproductive postdauer

adults (Bargmann and Horvitz 1991). When we incorporated these favorable conditions in the dauer recovery assay, we characterized *mut-7*(*pk720*) animals as *daf-r*.

Future experiments are needed to verify if RNAi pathways function in specific tissues to regulate the decision to recover from the dauer stage. We speculate that as seen in dauer formation (Chapter 2), RNAi functions in neurons to regulate dauer recovery decisions. ASJ neurons are good candidates to test this possibility as they have been implicated in dauer exit previously in addition to their role in dauer formation (Bargmann and Horvitz 1991). Our studies found that MUT-16 activity is required in ASJ for dauer formation in high pheromone conditions. It will be interesting to explore the role of MUT-7 in the molecular mechanism that surveys the environmental conditions for signaling the worms to recover from dauer. Future experiments could test whether there are parallel RNAi pathways functioning in different sets of neurons regulating dauer entry and dauer recovery. Mutants like *daf-2* or *daf-7* with known *daf-c* phenotypes can be tested alongside as controls (Malone and Thomas 1996).

Chapter 6

Discussion

6.1 Model

We propose a model where small RNA signals in neurons promote expression of G proteins that associate with neuron-specific receptors in order to modulate larval development in response to environmental cues. Our model is built on the following known facts and findings: *C. elegans* L1 larvae utilize their neurons to sense pheromone using their cilial endings (Albert *et al*. 1981, Perkins *et al*. 1986, Bargmann *et al*. 1991, Schackwitz *et al*. 1996, Vowels and Thomas 1992, Bargmann *et al*. 2006). We showed that this phenomenon is mediated by RNAi activity upstream (or parallel) to insulin-like and TGF- β pathways in subsets of neurons (ASI, ADL and ASJ) promoting dauer formation (Chapter 2, sections 2.3.2-2.3.4). We also found that the expression of *gpa-1*, *gpa-3*, and *gpc-1*, is significantly downregulated in *csr-1* and *mut-16* mutants compared to wild-type background (Chapter 2 section 2.3.5). *gpa-1*, *gpa-3*, and *gpc-1* genes code for G proteins associated with GPCRs required for sensation of pheromone in amphid neurons (Zwaal *et al*. 1997, Jansen *et al*. 1999, Lans and Jansen 2007, Kim *et al*. 2009, McGrath *et al*. 2011). Accordingly, we propose that *Mutator* complex generated siRNAs associating with CSR-1 AGO pathway positively regulate the expression of genes required for sensation of high pheromone (Fig. 21). Although we found RNAi function in neurons to be important for dauer formation in other stress conditions including temperature and starvation, the specific neurons where RNAi functions to regulate dauer formation in these conditions remain unidentified. Our model

is relevant to RNAi mechanism in high pheromone conditions and additional studies will need to be done to identify and characterize RNAi pathways regulating temperature and starvation induced dauer formation.

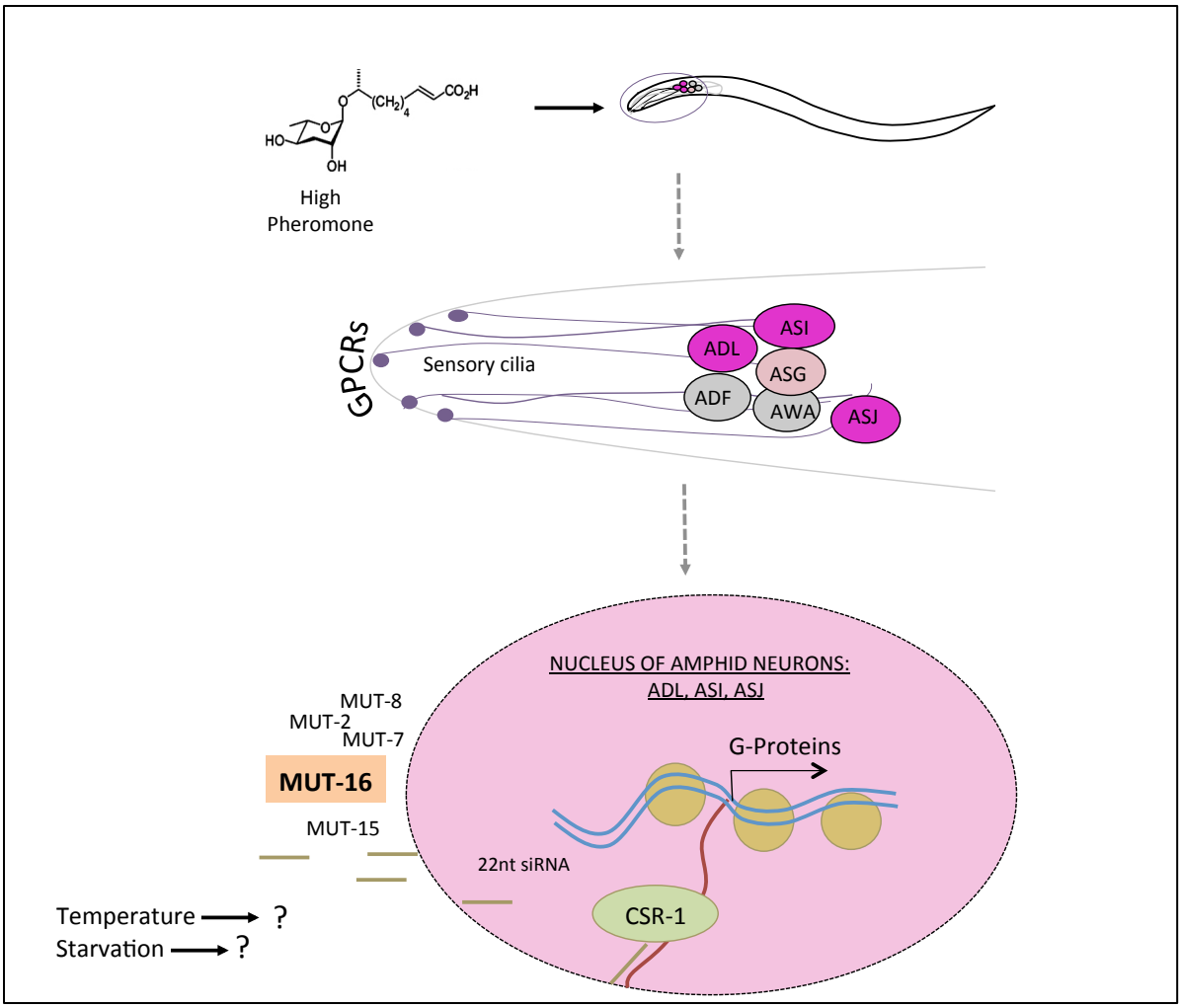


Fig. 21. Proposed model: RNAi pathways positively regulate transcription of genes required in amphid neurons for sensation of high pheromone to induce dauer formation.

6.2 RNAi in neurons is required for dauer formation in high pheromone stress

The main focus of this study is to investigate whether endogenous RNAi is playing a role in the regulation of dauer formation behavior in *C. elegans*. We showed that strains carrying mutations in *Mutator* proteins and the nuclear CSR-1 AGO exhibit *daf-d* phenotypes in response to high pheromone concentration. In addition, we provide evidence that MUT-16 functions in a subset of pheromone sensing neurons to promote dauer formation in high pheromone conditions. Our findings have implicated a new role of endo-siRNA in somatic cells, specifically neurons, in dauer formation.

Since the publication reporting that dsRNA has the ability to silence endogenous genes in 1998, much progress has been made defining the genetic pathways and gene targets of RNAi in *C. elegans* (Fire *et al*. 1998; Youngman and Claycomb 2014). Although at least half of the protein-coding genes in the *C. elegans* genome are targets of an RNAi pathway (Gu *et al*. 2009), we know little about how these pathways regulate endogenous genes, particularly in neurons. Previous work from our lab and others has shown that *Mutator* proteins and the nuclear AGO NRDE-3 play a role in regulating neuronal genes in response to environmental and developmental history. For example, stable down-regulation of the guanylyl cyclase gene *odr-1* in AWC neurons is required for experience-dependent olfactory adaptation to the odorant butanone, and is dependent upon MUT-7 and NRDE-3-mediated heterochromatin formation at the *odr-1* locus (Juang *et al*. 2013). Similarly, our recent work has shown that down-regulation of the *osm-9* TRPV channel gene in ADL neurons of animals that passed through the dauer stage is dependent upon a majority of the *Mutator* proteins and NRDE-3 AGO, resulting in altered responses to ascr#3 in adults (Sims *et al.* 2016). While the previous

examples illustrate how RNAi can silence neuronal genes in response to environmental history, our results indicate that MUT-16 and CSR-1 AGO are required in L1 larvae, regardless of their history, to promote transcription of genes with functions in sensory signaling of pheromone stress, including *gpa-1*, *gpa-3*, and *gpc-1*. These genes are expressed in ASI, ASJ, and ADL sensory neurons, among others, and loss-of-function mutations in these genes result in defects in dauer formation in response to pheromone (Zwaal *et al*. 1997, Jansen *et al*. 1999, Lans and Jansen 2007, Kim *et al*. 2009). Detection of high levels of a single ascaroside or ascaroside blends by a single neuron would be sufficient to induce dauer formation (Butcher *et al.* 2007). However, since we have not shown that the ASI, ASJ, and ADL neurons have pheromone-specific defects in *mut-16* and *csr-1* hypomorph strains, the possibility exists that these neurons have an overall reduced function in response to other stimuli as well.

6.3 A distributed chemosensory circuit promotes dauer formation in high pheromone concentrations

Our finding that expression of MUT-16 in a subset of individual sensory neurons rescues the *daf-d* phenotypes of *mut-16(pk710)* suggests that a distributed chemosensory circuit functions to detect high pheromone concentrations and promote dauer formation. Distributed neural circuits have been described previously in *C. elegans* as a mechanism for aggregation in response to low oxygen and pheromones exhibited by *npr-1(lf)* strains (Chang *et al.* 2006, Macosko *et al*. 2009). In addition, detection of chemical attractants at different concentrations, such as benzaldehyde and isoamyl alcohol, is distributed among a similar subset of chemosensory neurons (Yoshida *et al*. 2012, Leinwand *et al.* 2015). The idea that multiple neurons are working

to sense and induce dauer formation is not very surprising given that various GPCRs expressing in specific neurons have been characterized to sense individual pheromone molecules. What was unexpected was that we could rescue *mut-16(pk710)* dauer formation defects by expression of MUT-16 in ADL alone, which has not previously been implicated in pheromone detection during dauer formation. ADL has been shown to contribute to dauer formation in response to noxious chemicals, and exhibited a minor contribution to dauer formation when ablated with ASJ, suggesting that it might have a modulatory function for promoting dauer formation (Schackwitz *et al*. 1996, Neal *et al*. 2016). Alternatively, ADL may detect unknown molecules in crude pheromone that promote dauer formation. This result is consistent with the observation that expression of SRBC-64 and SRBC-66 in ASK alone can rescue the dauer formation defects of their respective null mutants, and contribute to the detection of ascr#1-3 (Kim *et al*. 2009). Based on our results, we speculate that detection of high pheromone concentrations by ADL, ASI, and ASJ results in downstream modulation of TGF-β and insulin signaling pathways to promote dauer formation. Further experimentation is required to test if expression of MUT-16 in ASK also rescues the *mut-16(pk710) daf-d* phenotypes as predicted by our model knowing that G protein GPA-3 expresses in ASK (Lans and Jansen 2007).

Furthermore, we propose that a distributed chemosensory circuit to promote dauer formation in the presence of high pheromone concentrations provides an adaptive advantage to *C. elegans* animals. The concentrations of the major ascaroside molecules isolated from *C. elegans* populations grown at 20°C differs from populations grown at 25°C, indicating that pheromone composition of crude dauer pheromone is

dependent upon environmental conditions (Jeong *et al*. 2005, Butcher *et al*. 2007, Butcher *et al*. 2008). Thus, the ability of *C. elegans* larva to detect a high concentration of any one ascaroside component in early development allows for phenotypic plasticity in an overcrowded area, regardless of other environmental conditions such as temperature. In this study, we provide evidence that RNAi pathways function early in the sensory signaling of high pheromone levels by promoting expression of G proteins in pheromone-sensing neurons. Although dauer-inducing environmental conditions are detected by a complex network of chemosensory neurons found in the head of worms, little is known about the concurrent integration of these signals. Since other animals, such as pea aphids, also form polyphenisms due to overcrowding, we predict that this work may have broader implications for the RNAi-mediated regulation of phenotypic plasticity.

6.4 RNAi activity in neurons is required for dauer formation in diverse environments

C. elegans is capable of detecting small changes in temperature up to 0.05°C (Ramot et al., 2008, Kimura et al., 2004, Clark *et al*. 2006). Hence, it is not surprising that a change in temperature can affect behavior including dauer formation, which is also demonstrated through our temperature assay results. Our results suggest that endo-RNAi is required for *C. elegans* to respond to environmental cues and enter the appropriate developmental trajectory. We found that different groups of RNAi mutants exhibited different *daf* phenotypes at 25°C and 27°C, and showed a distinct pattern from that seen in pheromone dauer formation assays. In order to determine if the molecular basis of this regulation is tissue specific we rescued *mut-16* RNAi mutant exclusively in

neurons and subjected the transgenic lines to temperature stress. The *daf-d* phenotype of the *mut-16*(*pk710*) was rescued at both the elevated temperatures suggesting that RNAi pathways in neurons are largely required for dauer formation irrespective of the degree of temperature stress.

The exact underlying molecular mechanisms and neurons detecting temperature to induce dauer formation in *C. elegans* are not well understood. *C. elegans* prefer to thrive in the temperature where they were cultivated. In case of temperature increase or decrease they manage to migrate to their cultivation temperature if accessible (Kuhara *et al*. 2008). This memory of the cultivation temperature or thermotaxis behavior requires a neural circuit involving AFD, which senses the temperature and activates downstream interneurons to integrate information regarding temperature change (Kuhara *et al*. 2008). Previous studies have also implicated molecular mechanisms involving guanynyl cyclase (*gcy-8*, *gcy-18*, *gcy-23*) and ion gate channels (*tax-2*, *tax-4* subunits) expressed in AFD neurons, with AWC neuron contributing in a modulatory role in thermosensory navigation behaviors (Biron *et al*. 2008, Kuhara *et al*. 2008, Garrity *et al*. 2010). We have not identified specific neurons wherein RNAi pathway is required for dauer formation at high temperatures. If the small endo-siRNA pathway is required for detection of high temperature similar to RNAi regulation of G protein genes in high pheromone conditions, we can rescue RNAi mutants in individual neurons and test them in high temperature conditions using dauer formation assays. Given that AFD and/or AWC are required for thermosensation, we could potentially rescue *mut-16*(*pk710*) in these specific neurons to elucidate the underlying RNAi mechanisms in thermosensation and dauer formation. Hence, further experiments are necessary to

determine how MUT-16 functions to regulate sensation of high temperatures to promote dauer formation.

The results of our starvation assays indicate that CSR-1 and a different subset of *Mutators* are required for starvation induced dauer formation than are needed in temperature and pheromone conditions. Based on our model, we predict that RNAi is required for starvation-induced dauer formation in the neurons that detect low food availability. Food availability is encoded by calcium/calmodulin-dependent protein kinase I (CamKI) in the AWC and ASI sensory neurons (Neal *et al*. 2015) although the chemical identity of the 'food signal' is yet to be identified. One of the well known 'food information' signals comes from the secretion of serotonin that is required for food odor associated learning in worms (Nuttley *et al*. 2002). How small RNAi pathways in neurons may facilitate detection of this information needs more investigation. Rescuing *mut-16* neuron specifically, perhaps in AWC, and subjecting them to starvation-induced dauer formation assay can identify individual neurons or subsets of them wherein RNAi activity is required for dauer formation in starvation conditions. Presence of food also promotes the exit from dauer stage and we have found that MUT-7 regulates dauer recovery decision (Chapter 5). It is possible that the inability of *mut-7* mutant strain to detect food availability resulted in *daf-r* phenotype. Properties of food signals are still being investigated to know the trigger for dauer recovery that might give more insight into this process (Kaul *et al*. 2014).

It is also worth noting that disruption of RNAi pathways can increase the incidence of incomplete dauer formation. Mutants that exhibit partial dauer phenotypes have been reported previously. In dauer inducing conditions, mutations in *daf-16*, *daf-*

18, and *daf-20* genes result in abnormal larvae where some tissues resemble that of a dauer while others looked L3-like (Vowels and Thomas 1992). The partial dauer phenotypes have been utilized in epistasis studies for ordering genes in dauer formation pathways (Vowels and Thomas 1992). Also, partial dauers exhibited alae formation, but had intermittent pharyngeal activity and were shorter in length.

Additionally, at 27°C dauer formation can be transient despite of prevalent continuous stress (Ailion and Thomas 2000). It is possible that at this temperature the RNAi mutants may show increased frequencies of transient dauer formation than wild-type. If this hypothesis is correct, then it is possible that RNAi pathway is also required to make dauer exit decision in high temperature conditions. Indeed, we have found MUT-7 to be important for dauer recovery in high pheromone conditions (Chapter 5). The mechanisms intrinsic to temperature induced dauer formation and exit are largely unknown that need to be further studied in order to address these questions.

6.5 Applications of our studies in understanding phenotypic plasticity

6.5.1 RNAi is required for expression of polyphenism

One of the first identified functions of RNAi was its role in defense against viruses and transposable elements that became part of extensive host-parasite studies in eukaryotic animals (Reviewed in Matranga *et al*. 2007). In plants, changes in the epigenetic state allow transposon and retroelement movement that facilitates and increases the plants' ability to survive in harsh conditions (Miura 2001, Tsukahara 2009, Reinders 2009). For instance, *C. plantagineum* have allowed increased production of a repertoire of small RNA to be generated in stressful conditions against desiccation by activating ABA pathways (Hilbricht 2008). A study conducted in *Drosophila* has also

looked at the role of RNAi as part of their immune system and found RNAi pathway genes for driving extensive adaptive evolution in response to pathogens (Obbard *et al*. 2009). In this study, we demonstrated the requirement of the RNAi pathway in dauer formation, which is an adaptive stage in *C. elegans* development. Our study is yet another example of how RNAi pathway can impact organisms' ability to cope with their environment, and we have shown that the underlying mechanism to this coping strategy is stress specific. Our findings aim to improve our knowledge of the ability of RNAi pathway to control genes relating to metabolism, development, and behavior in response to stress, which would be an important factor for increasing the scope for phenotypic plasticity and ultimately allow polyphenism to flourish.

6.5.2 Stress dependent epigenetic marks can generate diverse phenotypes

Our work indicates that pheromone, temperature, and starvation induced dauer formation may have distinct molecular signatures. Our visual observations suggest that the dauers induced by different stresses might have physiological differences in addition to the typical features of dauers. We noted that pheromone induced dauers typically have a noticeably long, hardy, constricted, and retractile body. As reported before, temperature induced dauers were paler and fragile looking, and needed careful examination of the pharynx under the microscope (Ailion and Thomas 2000). 27°C-induced dauers were also less responsive when prodded compared to pheromone-induced dauers. In depleted food conditions, dauers are shorter and exhibit intermediate cuticle thickness compared to pheromone and temperature-induced dauers. These observations suggest that dauers induced by distinct stresses are physiologically different.

If the differences in the molecular pathways leading to dauer formation are reflected in the phenotypes giving rise to different 'types of dauers', then further studies would open up new opportunities to understanding the underlying mechanisms being adopted in humans (and other animals) in response to various types of stress. The possibility that each type of stress can result in distinctive epigenetic marks is very exciting and can help us solve many issues related to both mental and even physical trauma. This is also a very intriguing avenue in forensic sciences, which is already using epigenetic tools extensively to perform studies (Reviewed in Courts and Madea, 2010 and Silva *et al*. 2015).

The focus of this dissertation is to understand the role of RNAi pathways in regulating dauer formation and elucidating a possible mechanism of stress perception via neurons right at the onset of stress. It would be fascinating to know if environmental changes can trigger and set molecular marks in single neurons that are established and maintained into adulthood. Our studies have started to unravel the intricate pathways regulating dauer formation at single neuron level and study the importance of components of RNAi pathways like CSR-1 AGO and MUT-16 in this process. Given the similarity in hormonal signaling and RNA interference pathways in *C. elegans* and other biological systems (Chapter 1- section 1.3), including humans, we believe this study answers crucial questions regarding effects of stress and will help us understand how to encounter neurodegenerative diseases like schizophrenia and depression which are very relevant today (Bale *et al*. 2010, Felitti *et al.* 1998, Dube *et al.* 2001).

6.6 Future Directions

Our work demonstrates the need of RNAi pathway component MUT-16 in individual chemosensory neurons for facilitating the sensation of pheromone stress resulting in dauer formation. Although we found that MUT-16 activity in neurons is generally sufficient for dauer formation irrespective of type of stress induced, specific neurons or subsets of neurons where RNAi pathway is regulating sensation of high temperature and starvation are yet to be identified. Neurons namely AWA, ASJ, and ASI are required for food signal integration and transduction in dauer formation (Neal *et al*. 2015). Additionally, AFD and AWC neurons show modified states during dauer stage and are implicated in temperature dependent behaviors (Albert and Riddle 1983, White *et al*. 1986, Biron *et al*. 2008). Hence, more *mut-16* neuron specific rescues can be generated and tested to know if these and other neurons are required for sensation of food signals and temperature changes. Therefore, our approach of testing neuron specific rescues could be used to map out all the neurons where RNAi is required for dauer formation. Additionally specific CSR-1 targets that regulate dauer formation genes can be identified, which will give us an insight into not only how the small RNA signals are initiated upon sensation of environmental stress, but also how the signals are regulating dauer formation genes in intra-or intercellular manner. Furthermore, the site of action of more *Mutators* could also be tested to know if the *Mutators* including MUT-16 function together in the soma, as seen in the germ line, to regulate dauer formation (Phillips *et al*. 2012). Besides these studies, our observations suggest that all dauers are not created equal. The morphology of dauers and partial dauers induced by the three stresses can be investigated further followed by a closer look at their

molecular states, *i.e*., epigenetic states. In fact, ongoing studies in our lab have suggested that different types of stresses leave their own ‘signatures’ in postdauer adults and RNAi pathways are required maintain these differences. Together, an overall understanding of RNAi function right at the start at stress perception level to manifestation of polyphenism in *C. elegans* will improve our understanding of this phenomenon in humans and other biological systems as well.

Appendix 1

Strains used in this study

WM30 [*mut-2*(*ne298*)I]

NL1820 [*mut-7*(*pk720*)III]

WM29 [*mut-8*(*ne221*)I]

NL1838 [*mut-14*(*pk738*)]

GR1747 [*mut-15*(*tm1358*)V]

GR1823 [*mut-16*(*mg461*)I]

NL1810 [*mut-16*(*pk710*)I]

NL2098 [*rrf-1*(*pk1417*)I]

NL2099 [*rrf-3(pk1426)*II]

YY158 [*nrde-3*(*gg66*)X]

WM193 [*csr-1*(*tm892*)IV;*neIs20*{*pie-1*::3xFLAG::*csr-1+unc-199*(+)}]

GR1311 [*daf-3*(*mgDf90*)X]

WM206 [*drh-3*(*ne4253*)I]

WM191 [mago12]{Full genotype: *sago-2*(*tm894*) *ppw-1*(*tm914*) *ppw-2*(*tm1120*) *wago-2*(*tm2686*) *wago-1*(*tm1414*) I; *wago-11*(*tm1127*) *wago-5*(*tm1113*) *wago-4*(*tm1019*) II; *hrde-1*(*tm1200*) *sago-1*(*tm1195*) III; *wago-10*(*tm1186*) V; *nrde-3*(*tm1116*) X}

CB1370 [daf-2(e1370)III]

CB1372 [*daf-7*(*e1372*)III]

NL3321 [*sid-1*(*pk3321*)V]

HC196 [*sid-1(qt9)* V]

SH236 [*mut-16*(*pk710*); *pdrEx64(rab-3*p::*mut-16*::*gfp*)]

SH240 [*mut-16*(*pk710*); *pdrEx63(rab-3*p::*mut-16*::*gfp*)]

SH308 *mut-16*(*pk710*); *pdrEx83*(*ges-1*p::*mut-16*::*gfp; unc-122*p*::dsRed*)

SH309 *mut-16*(*pk710*); *pdrEx84*(*ges-1*p::*mut-16*::*gfp; unc-122*p*::dsRed*)

SH273 [*mut-16*(*pk710*); *pdrEx65(trx-1*p::*mut-16*::*gfp*)]

SH278 [*mut-16*(*pk710*); *pdrEx66(trx-1*p::*mut-16*::*gfp*)]

SH284 [*mut-16*(*pk710*); *pdrEx67*(*unc130p*::*mut-16*::*gfp*)]

SH285 [*mut-16*(*pk710*); *pdrEx68*(*unc130p*::*mut-16*::*gfp*)]

SH286 [*mut-16*(*pk710*); *pdrEx69*(*gpa-4p*::*mut-16*::*gfp*)]

SH287 [*mut-16*(*pk710*); *pdrEx70*(*gpa-4p*::*mut-16*::*gfp*)]

SH288 [*mut-16*(*pk710*); *pdrEx71*(*sre-1p*::*mut-16*::*gfp*)]

SH289 [*mut-16*(*pk710*); *pdrEx72*(*sre-1p*::*mut-16*::*gfp*)]

SH277 [*mut-16*(*pk710*); *pdrEx73*(*srh-142p*::*mut-16*::*gfp*)]

SH290 [*mut-16*(*pk710*); *pdrEx74*(*srh-142p*::*mut-16*::*gfp*)]

SH291 [*mut-16*(*pk710*); *pdrEx75*(*odr-10p*::*mut-16*::*gfp*)]

SH292 [*mut-16*(*pk710*); *pdrEx76*(*odr-10p*::*mut-16*::*gfp*)]

SH293 [*mut-16*(*pk710*); *daf-2*(*e1370*)]

SH294 [*mut-16*(*pk710*); *daf-7*(*e1372*)]

SH296 [*mut-16(pk710)* I; *csr-1(tm892)* IV/ *nT1(qIs51*) (IV, V)]

SH48 [N2;K09E9.3P::int4]

Appendix 2

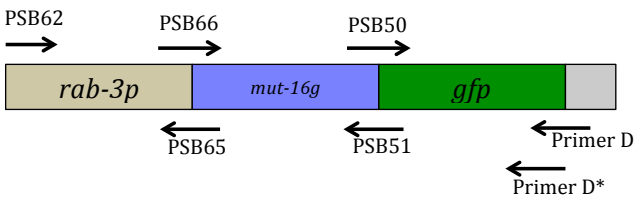
Primer sequences used for cloning pan-neuronal and neuron-specific rescue constructs of *mut-16.* Cloning strategies and primer D and D* sequences are based on Hobert 2002.

Note: Primer sequences used for cloning of ASI and ADL neuron specific rescue constructs of *mut-16* is listed in Sims *et al.* 2016.

Note: *mut-16* gene sequences are highlighted in **bold** and *gfp* sequences are highlighted in green.

Primers for *rab-3*p*::mut-16::gfp*

	Product	Primer name		Sequence
PCR1	*rab-3 promoter*	PSB62	Fw	GATTTCGGTCCTGGTGCAGC
		PSB65	Rev	**GATAATCATCATCACTTTCGGACAT** GGTCTTCTTCGTTTCCGCC
PCR2	*mut-16 gene*	PSB66	Fw	GGCGGAAACGAAGAAGACC**ATGTCC GAAAGTGATGATGATTATC**
		PSB51	Rev	GTTCTTCTCCTTTACTCAT**GTTTCGG ATATCATCTTTCAAAACG**
PCR3	*gfp*	PSB50	Fw	**CGTTTTGAAAGATGATATCCGAAAC** ATGAGTAAAGGAGAAGAAC
		D	Rev	AAGGGCCCGTACGGCCGACTAGTAGG
PCR4	*mut-16::gfp*	PSB66	Fw	Listed above
		D	Rev	Listed above
Final PCR	*rab-3*p*::mut-16::gfp*	PSB62	Fw	Listed above
		D*	Rev	GGAAACAGTTATGTTTGGTATATTGGG

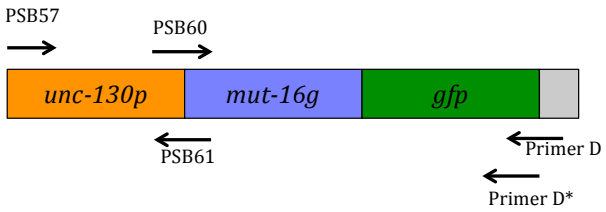


Appendix 2—Contd.

*** *rab-3*p*::mut-16::gfp* inserted into TOPO XL vector (Invitrogen) was used as a template to amplify *mut-16::gfp* fragment for PCRs below.**

Primers for *unc-130*p*::mut-16::gfp*

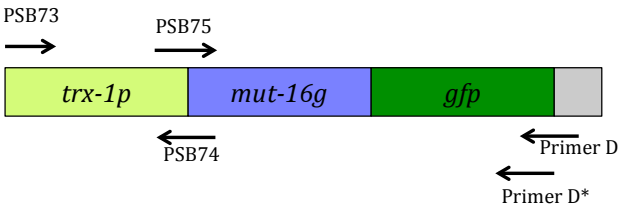
	Product	Primer name		Sequence
PCR1	*unc-130 promoter*	PSB57	Fw	GCCACCCAGAATTAGTCGATT
		PSB61	Rev	**CATCATCACTTTCGGACAT**TG TTACCGGTGTCTACCTAGTTAG
PCR2*	*mut-16::gfp*	PSB60	Fw	CTAACTAGGTAGACACCGGTAAC AATGTCCGAAAGTGATGATG
		D	Rev	Listed Above
Final PCR	*unc-130*p*::mut-16::gfp*	PSB57	Fw	Listed above
		D*	Rev	Listed above



Primers for *trx-1*p*::mut-16::gfp*

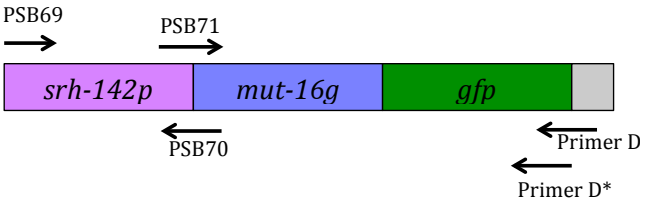
	Product	Primer name		Sequence
PCR1	*trx-1 promoter*	PSB73	Fw	GACCTTGGTAACCTCGACTTTG
		PSB74	Rev	**GATAATCATCATCACTTTCGGACAT**GAT GAAATACAAGTGTAGAAAATTC
PCR2*	*mut-16::gfp*	PSB75	Fw	GAATTTTCTACACTTGTATTTCAT CATGTCCGAAAGTGATGATGATTATC
		D	Rev	Listed above
Final PCR	*trx-1*p*::mut-16::gfp*	PSB73	Fw	Listed above
		D*	Rev	Listed above

Appendix 2—Contd.



Primers for *srh-142*p*::mut-16::gfp*

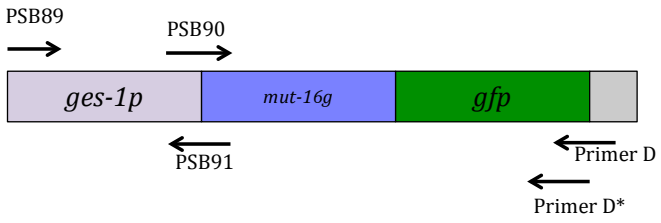
	Product	Primer name		Sequence
PCR1	*srh-142 promoter*	PSB69	Fw	CAAATGTTGATATGTTTTCCCGAG
		PSB70	Rev	**GATAATCATCATCACTTTCGGACAT** ATTGGCAAAAAGAAAAAAGAGGTG
PCR2*	*mut-16::gfp*	PSB71	Fw	CACCTCTTTTTTCTTTTTGCCAA TATGTCCGAAAGTGATGATGATTATC
		D	Rev	Listed above
Final PCR	*srh-142*p*::mut-16::gfp*	PSB69	Fw	Listed above
		D*	Rev	Listed above



Appendix 2—Contd.

Primers for *ges-1*p*::mut-16::gfp*

	Product	Primer name		Sequence
PCR1	*ges-1* promoter	PSB89	Fw	GGCTCTCTTTCAGATCACTTAAAAC
		PSB91	Rev	**GATAATCATCATCACTTTCGGA** CATCTGAATTCAAAGATAAGATATG
PCR2*	*mut-16::gfp*	PSB90	Fw	CATATCTTATCTTTGAATTCAG ATGTCCGAAAGTGATGATGATTATC
		D	Rev	Listed above
Final PCR	*ges-1p::mut-16::gfp*	PSB89	Fw	Listed above
		D*	Rev	Listed above



Appendix 2—Contd

Primer sequences used for qRT-PCR experiment

y45f10d.4	Forward	5' ACTTCAGTTGCTCAGTATCACG
	Reverse	5' TCATGACATCTCCACAAGCTG
gpa-1	Forward	5' TTGCATTTGAGTCATTTAGTGGAC
	Reverse	5' TCTCTTTGTTCTCTGCCATACTTC
gpa-3	Forward	5' GCCGAGAAGATTAAAAGAACTTCA
	Reverse	5' AACTTTTCCTCAATATATCGACACG
gpc-1	Forward	5' GCTGAGGCCAACATTCAAC
	Reverse	5' GCATGTCGTTGGTCTTGTTC
flp-21	Forward	5' TGAACGCATATTTGGGTTT
	Reverse	5' CCGAGACCACGTTTCATTG

Appendix 3

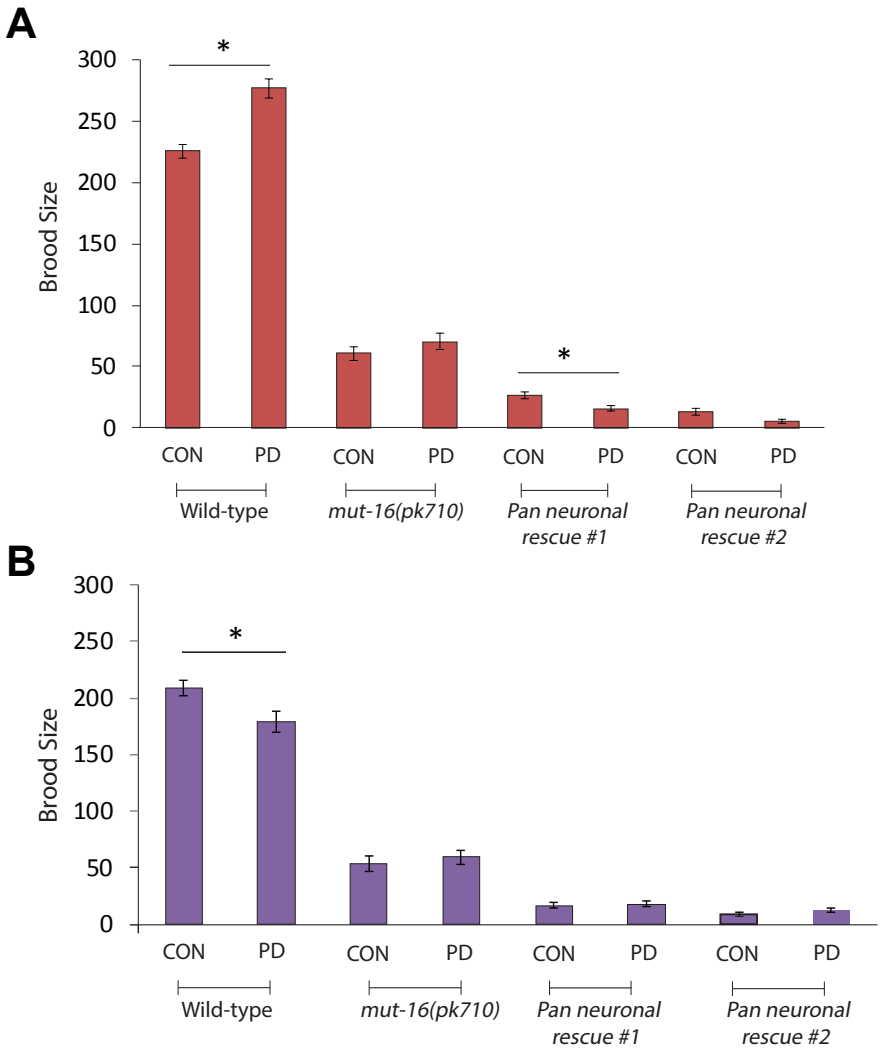


Fig. S1. Brood size assays were conducted using *mut-16* pan neuronal rescues to study the effect of mut-16 expression in neurons on brood size. **A**. Brood size comparison for *mut-16* pan-neuronal rescue controls (CON) and post dauers (PD_{Pher}). * indicates $p<0.05$ using student's T-Test. N=3. **B**. Brood size comparison of *mut-16* pan-neuronal rescue controls (CON) and post dauers (PD_{starve}) * indicates $p<0.05$. N=3. Error bars represent SEM.

Our studies have found that endogenous RNAi pathways, specifically *mut-16*, functions in neurons cell-autonomously and that siRNA signals may not propagate to other tissue types in dauer formation (Fig. 8 and Fig. 9). However, we wanted to test if

the molecular signals generated in the neurons can contribute to postdauer phenotypes as a result of passage through dauer. Therefore, we extended our studies to determine if neuronal MUT-16 expression could affect other life history traits in *C. elegans* adults. We chose to test the brood size of *mut-16* pan-neuronal rescues given our initial observations that these strains (same lines used for dauer formation assays) exhibit sterile phenotypes.

Brood size in postdauer adults (PD) is different depending on early-experienced stress. Mutations in *mut-16* eliminate changes in PD/CON brood sizes for both stresses. The possibility exists that stress-specific changes in brood size result from propagation of small RNA signals generated from sensory neurons that detect the dauer-inducing stress. Additionally, a previous study provides evidence for the generation of small RNA signals in neurons that are transmitted to the germline resulting in transgenerational silencing (Devanapally *et al*. 2015). Thus, we hypothesized that rescue of *mut-16* expression in neurons could impact the brood size in adults. We generated postdauers via high pheromone (PD_{Phe}) and starvation stress (PD_{stv}). We counted the brood size of controls (animals that do not experience stress) and postdauers for wild-type, *mut-16(pk710)*, and pan neuronal rescues according to the methods described in Hall *et al*. 2010.

Wild-type PD_{Pher} produced more progeny compared to their corresponding controls as reported before (Hall *et al.* 2010) (Fig. S1A). *mut-16*(*pk710*) controls (60.9 ± 5.6) and *mut-16* (*pk710*) postdauers (70.4 ± 6.6) produced similar numbers of progeny confirming previous results (Hall *et al.* 2013). This finding implies that MUT-16 is required for regulating the brood size differences between controls and postdauers in

wild-type. In addition, we observed that the PD/CON changes in brood size are not rescued in the pan-neuronal rescues (CON#3- 26.7 ± 2.7; PD#3- 15.7 ± 2.2; CON#4- 13.4 ± 3.0; PD#4- 5.8 ± 1.7) but exhibit significant reduction ($p<0.0005$, using two tailed T-test, comparing rescues to wild-type and *mut-16*(*pk710*)) in the brood size compared to wild-type (CON- 225.4 ± 5.8; PD- 276 ± 7.8) and *mut-16*(*pk710*)(CON- 60.9 ± 5.6; PD- 70.4 ± 6.6) for both controls and postdauers. These results show that since pan-neuronal rescue lines are essentially overexpressing MUT-16, excess RNAi activity in neurons could have adverse effects on the brood size.

Likewise, we subjected pan-neuronal control and postdauer adults (PD_{stv}) obtained via starvation to brood size assays (Fig. S1B). As observed previously, wild-type postdauers (178.8 ± 8.8) produced fewer progeny than controls (209.2 ± 6.7)(M. Ow and A. Nichitean, personal communication). Similar to pheromone stress, the *mut-16* mutation eliminated the brood size differences between wild-type controls and postdauers (Fig S1B). The pan-neuronal rescues consistently produced fewer progeny (CON#3- 16.8 ± 2.8; PD#3- 17.8 ± 2.5; CON#4- 8.8 ± 1.8; PD#4- 12.2 ± 2.0) compared to wild-type and *mut-16*(*pk710*)(CON#3- 53.6 ± 6.7; PD#3- 59.5 ± 5.9) strains, and there was insignificant difference between rescue controls and postdauers. These results suggest that while *mut-16* rescue in neurons does not restore the differences between PD/CON brood size, neuronal amplified siRNAs can impact germline development and brood size. Further experiments are required to test the mechanism of how small RNAs are transported from neurons to the germline to impact the brood size.

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EDUCATION

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M.S., **Summer 2010** — Department of Biological Sciences, University of Alabama in Huntsville, AL. Advisor: Dr. Gopi Podila.
B.E., **Spring 2007** — Biotechnology, New Horizon College of Engineering (VTU), Bangalore, India.

PEER REVIEWED PUBLICATIONS

1. Bharadwaj, Pallavi S., and Sarah E. Hall. "Endogenous RNAi Pathways Are Required in Neurons for Dauer Formation in *Caenorhabditis elegans*." Genetics (2017): genetics-116. PMID: 28122825

POSTERS

1. Poster: "Endogenous RNAi Pathways Regulate Dauer Formation in *C.elegans*", Pallavi Bharadwaj and Sarah Hall. In Aging, Metabolism, Stress, Pathogenesis and Small RNAs in *C.elegans* Conference at University of Wisconsin, Madison, WI, Jul 2016.
2. Poster: "Endogenous RNAi Pathways Regulate Dauer Formation in *C.elegans*", Pallavi Bharadwaj and Sarah Hall. In CNY *C.elegans* Meeting, Syracuse, NY, Apr 2015 and Apr 2014.
3. Poster: "Analysis of Methylation Patterns and Chromatin Modifications in Different Genotypes of Poplar Trees.", Pallavi Bharadwaj, Leland Cseke and Gopi Podilla. In HudsonAlpha Institute of Biotechnology, Spring Symposium at Huntsville, AL, Mar 2010.

AWARDS & RECOGNITION

1. GSO travel grant from Graduate Student Organization at Syracuse University, Apr 2016.
2. Graduate Summer Fellowship from Department of Biology at Syracuse University, Summer 2014.
3. University Peak of Excellence, Graduate Research Fellowship at Auburn University, Aug 2011 to May 2012.
4. Academic recognition for securing First Class with Distinction in B.E, Jul 2007.

TEACHING EXPERIENCE

Teaching Assistant, Department of Biology, Syracuse University, NY — Fall 2011 to Fall 2016

- Genetics Lab (BIO 435), Fall 2014, Fall 2015 and Fall 2016.
- Integrative Biology Lab (BIO 305), Spring 2014, Spring 2015 and Spring 2016.
- General Biology I (BIO 121), Fall 2011 and Fall 2013.
- Molecular Biology Lab (BIO 465), Spring 2012, Fall 2012, Spring 2013.

Teaching Assistant, Department of Biological Sciences, University of Alabama in Huntsville, AL — Fall 2008 to Summer 2010

- Principles of Biology (BYS 119), Fall 2008 to Summer 2010.
- Tutor for UG Students, Department of Biological Sciences, University of Alabama in Huntsville, Summer 2009.

PROFESSIONAL SOCIETIES

- Genetics Society of America

ORGANIZATIONAL ACTIVITIES

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MENTORING EXPERIENCE

1. Austin Hager, UG trainee in Dept. of Biology at Syracuse University. Fall 2015 to Spring 2016. Now a medical student at Lake Erie College of Osteopathic Medicine.
2. Mailyn Nishiguchi, UG trainee in Dept. of Biology at Syracuse University. Summer 2014. Now a PhD student in the Dept. of Biology, University of Pennsylvania.
3. Nicole Darling, High school student trainee in Dept. of Biology at Syracuse University, Fall 2014 to Summer 2015.
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