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 or behavior. In the nematode, *Caenorhabditis elegans*, environmental conditions perceived early in development determine whether the animal enters either the reproductive cycle, or enters into an alternative diapause stage named dauer. Here, we show that endogenous RNAi pathways play a role in dauer formation in crowding (high pheromone), starvation, and high temperature conditions. Disruption of the *Mutator* proteins or the nuclear Argonaute CSR-1 result in differential dauer-deficient phenotypes that are dependent upon the experienced environmental stress. We provide evidence that the RNAi pathways function in chemosensory neurons for dauer formation, upstream of the TGF-β and insulin signaling pathways. In addition, we show that *Mutator* MUT-16 expression in a subset of individual pheromone-sensing neurons is sufficient for dauer formation in high pheromone conditions, but not in starvation or high temperature conditions. Furthermore, we also show that MUT-16 and CSR-1 are required for expression of a subset of G proteins with functions in the detection of pheromone components. Together, our data suggest a model where *Mutator*-amplified siRNAs that associate with the CSR-1 pathway promote expression of genes required for the detection and signaling of environmental conditions to regulate development and behavior in *C. elegans*. This study highlights a mechanism whereby RNAi pathways mediate the link between environmental stress and adaptive phenotypic plasticity in animals.

KEYWORDS dauer; RNAi; *Mutators*; CSR-1; *Caenorhabditis elegans*

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). Sex determination in alligators (Woodward and Murray 1993), caste determination in insects (Nijhout 1998, 1999), seasonal differences in phenotypes of adult butterflies *Precis Almana* (Nijhout 1999), and growth of a "helmet-like" structure in *Daphnia pulex* (water flea) in the presence of predators (Brewer *et al.* 1999) are examples of polyphenisms in response to unfavorable environmental conditions.

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; Hunt *et al.* 2010; Bonasio 2012; Humann *et al.* 2013). For example, in pea aphids, unwinged and winged morphs develop in response to favorable or unfavorable environments, respectively (Müller *et al.* 2001; Brisson 2010). Although genetically identical, winged and unwinged female populations exhibit differential DNA methylation patterns and transcriptional profiles of genes implicated in wing polyphenism (Brisson *et al.* 2007, 2010; 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.

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C. elegans is an excellent model system in which to study the molecular mechanisms regulating polyphenism as their developmental trajectory is determined by the environmental conditions experienced after hatching. Under favorable growth conditions, worms proceed through four larval stages (L1, L2, L3, and L4) to become reproductive adults. Under unfavorable conditions, such as low food availability, high temperatures, or high pheromone concentrations, L1 larvae will enter an alternative developmental stage called dauer (Cassada and Russell 1975; Golden and Riddle 1982; 1984a,b,c). Dauer larvae are stress-resistant and nonaging, and thought to facilitate dispersal in environmental conditions unfavorable for reproduction (Klass and Hirsh 1976; Larsen 1993; Frézal and Félix 2015). Once conditions improve, animals will exit dauer and resume continuous development as L4 larvae. Although adults that passed through, or bypassed, the dauer stage appear morphologically similar, we previously showed that *C. elegans* retain a cellular memory of their developmental history that is reflected in changes in gene expression, genome-wide chromatin states, and life history traits (Hall *et al.* 2010). Furthermore, we have shown that RNAi pathways are a major contributor to developmental history-dependent phenotypes in adults (Hall *et al.* 2013). However, the role of RNAi pathways in regulating environmentally induced phenotypic plasticity during early larval stages is unknown.

Environmental cues sensed by G protein coupled receptors (GPCRs) residing in the ciliary endings of sensory neurons differentially regulate the TGF-β and insulin signaling dauer formation pathways (Fielenbach and Antebi 2008). Mutations in genes operating in these signaling pathways can result in dauer constitutive (*daf-c*) or dauer deficient (*daf-d*) phenotypes (Riddle *et al.* 1981; Vowels and Thomas 1992). Animals that form significantly fewer dauer larvae than wild-type in response to environmental stress are considered *daf-d*, while *daf-c* animals can form dauers even in the absence of environmental stress. The primary cue for dauer entry in *C. elegans* is high local concentrations of dauer pheromone, which is a blend of hydrophilic ascaroside (ascr) molecules containing three to nine carbon side chains (Golden and Riddle 1982, 1984a,b,c; Jeong *et al.* 2005; Butcher *et al.* 2007, 2008). Exposure to unfavorable environmental conditions has been shown to inhibit DAF-7 TGF-β production in ASI sensory neurons during the L1 larval stage, resulting in dauer formation (Ren *et al.* 1996; Schackwitz *et al.* 1996). In addition, unfavorable conditions can result in diminished insulin signaling, causing reduced expression of *daf-28*/insulin in ASI and ASJ neurons during the L1 and dauer stages and *ins-6*/insulin in ASI neurons during the L2d stage (Li *et al.* 2003; Cornils *et al.* 2011; Neal *et al.* 2015). These two pathways converge onto the DAF-12 steroid hormone receptor, which acts a master regulator of dauer formation programs (Riddle *et al.* 1981; Vowels and Thomas 1992; Thomas *et al.* 1993; Antebi *et al.* 2000).

In this study, we sought to characterize the role of endogenous RNAi pathways in the regulation of dauer formation in *C. elegans*. In worms, small interfering RNAs (siRNAs) are characterized by their biogenesis and associated Argonautes (AGOs). Primary siRNAs are low in abundance, 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), 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; Maine *et al.* 2005; Vought *et al.* 2005; Aoki *et al.* 2007; Pak and Fire 2007; She *et al.* 2009; Gent *et al.* 2010; Vasale *et al.* 2010; Pak *et al.* 2012). In addition, a group of proteins called the *Mutators* were 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 much progress has been made in characterizing the biogenesis of endogenous siRNAs, little is known about how RNAi pathways target and regulate endogenous genes.

Here, we show that endogenous RNAi pathways are required for dauer formation in adverse environmental conditions. Mutations in the *Mutator* proteins and nuclear AGO CSR-1 pathway result in differential *daf-d* phenotypes that are dependent upon the experienced environmental stress. *Mutator* protein, MUT-16, acts in sensory neurons, likely upstream of the TGF-β and insulin signaling pathways, for dauer formation in high pheromone, starvation, or high temperature conditions. Our results suggest that MUT-16 and CSR-1 function to positively regulate expression levels of genes with sensory signaling functions, mediating the link between RNAi and formation of polyphenism in stressful environments in *C. elegans*.

Materials and Methods

C. elegans strains and maintenance

All worm strains were cultivated on NGM plates with *Escherichia coli* OP50 as the food source at either 15 or 20° according to standard methods (Brenner 1974; Stiernagle 2006). Worm strains used in this study are listed in Supplemental Material, File S1. Due to the propensity of high spontaneous mutation through transposon mobilization in a subset of the RNAi strains used (*i.e.*, *Mutators*) (Vastenhouw *et al.* 2003), we limited the number of propagated generations after thawing, and genetically backcrossed strains to wild-type when necessary.

Dauer formation assays

Different dauer formation assays were used to test *daf* phenotypes under high pheromone, starvation, or high temperature conditions. To test dauer formation in the presence of high pheromone concentrations, we conducted assays using

crude pheromone as previously described with a few modifications (Neal *et al.* 2013; Zhang *et al.* 2013). For each independent batch of crude pheromone, one activity unit was defined as the amount of pheromone that resulted in 33% dauers in wild-type animals (Zhang *et al.* 2013). Each dauer formation plate contained four activity units of pheromone that were mixed into the medium during plate preparation. Water was used instead of pheromone for the control plates. Each assay plate was seeded with 20 μl of 8 mg/ml (0.16 mg) *E. coli* OP50 that was heat-killed by incubating at 95° for 30 min.

Next, five well-fed 2-day-old adults were placed on assay plates for 3–6 hr at room temperature until ~60–80 eggs were laid on the plates, after which the adults were removed. For mutant strains that exhibited severe sterility phenotypes [*mut-2(ne298)*, *mut-7*(*pk720*), *csr-1* hypomorph, *csr-1(tm892)/nT1(qIs51)*, *mut-16*(*pk710*); *csr-1(tm892)/nT1(qIs51)*, and *drh-3(ne2453)*], eight adults were placed on the assay plates, and the amount of heat-killed *E. coli* OP50 was doubled to prevent starvation during the extended egg-laying period. No significant change in dauer formation was observed for wild-type animals using these modified conditions (Student's *t*-test, $P = 0.17$). The assay plates were incubated at 25° for 3 days, and then scored for dauers. For strains carrying a chromosome balancer, the *daf* phenotype of only non-GFP progeny was scored.

To perform dauer formation assays using high temperatures, a similar protocol was used as described above, with a few modifications. First, assay plates were prepared without pheromone. Second, mutant embryos were allowed to hatch at room temperature for 10–12 hr before transfer to 25 or 27° to prevent embryonic lethality or L1 diapause. Assay plates were scored for the presence of dauers after 4 days.

Starvation assays were conducted using assay plates prepared without pheromone that were seeded with 0.04 mg of heat-killed *E. coli* OP50. Embryos were allowed to hatch at room temperature before transfer to 25°. Dauer formation was scored after 5 days.

For all assays, dauers were distinguished from nondauers by the presence of pharynx pumping and dauer alae as previously described (Cassada and Russell 1975; Popham and Webster 1979; Albert and Riddle 1988; Riddle and Albert 1997; Hu 2007). For mutants that exhibited an increased number of L2d larvae or partial dauers, assay plates were re-examined after 24 hr (File S2). The progeny of all the animals assayed were scored at the same time. All dauer formation assays were performed in duplicate, in at least three biological replicates (File S1). Statistical significance of data were determined using one-way ANOVA with LSD or Tukey's HSD *post hoc* tests using SPSS (v. 23).

MUT-16 rescues

Genomic DNA template was used to amplify the full-length *mut-16* gene. The following tissue-specific promoters were used: *rab-3* (pan-neuronal), *ges-1* (intestine), *trx-1* (ASJ), *unc-130* (ASG), *gpa-4* (ASI), *sre-1* (ADL), *srh-142* (ADF), and *odr-10* (AWA) (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; Lanjuin *et al.* 2003; Miranda-Vizuete *et al.* 2006).

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, 4, and 1 ng/μl, respectively. *Unc-122*p::*dsRed* (30 ng/μl) was used as the coinjection marker. Two independent transgenic lines were used in all dauer formation assays. See File S3 for primer sequences.

RNA preparation and reverse transcription-real time PCR

Embryos were obtained by bleaching well-fed adult hermaphrodites as described by 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 (Life Technologies) according to the manufacturer's protocol, and reverse transcribed using SuperScript IV Reverse Transcriptase (Life Technologies). Quantitative real-time PCR was performed in triplicate using iTaq Universal SYBR Green Supermix (Bio-Rad) using a Bio-Rad CFX Connect Real-Time System. Normalization of gene expression for *flp-21*, *gpa-1*, *gpa-3*, and *gpc-1* was done using the mRNA levels of *y45f10d.4*, a somatically expressed gene that is not a documented CSR-1 target (Claycomb *et al.* 2009), and does not experience gene expression changes due to dauer-inducing conditions (M. C. Ow, K. Borziak, S. Dorus, S. E. Hall unpublished results). Primer sequences are listed in File S3.

DiI staining

Well-fed wild-type and *mut-16*(*pk710*) young adults were washed with M9 buffer (Stiernagle 2006), and transferred into 1.5 ml microcentrifuge tubes. Worms were washed in M9 buffer, and resuspended in M9 buffer containing 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) at 1:200 dilution prepared from a 2 mg/ml solution dissolved in *N*, *N*-dimethylformamide (Sigma) as previously described (Shaham 2006). The animals were rotated in the dark at room temperature for ~1 hr, and imaged using a Leica DM5500 B microscope and ORCA-R2 Digital C10600 camera (Hamamatsu).

Data availability

The worm strains used in this study are listed in File S1 and are available on request. The primer sequences used for cloning and qPCR experiments are listed in File S3.

Results

Endogenous RNAi pathways are required for dauer formation

In order to characterize how RNAi pathways regulate polyphenism in response to environmental stress, we conducted dauer formation assays using strains carrying mutations in a subset of genes with functions in endogenous RNAi pathways. First, we subjected newly hatched larvae to high concentrations of crude dauer pheromone as previously described, and scored the percentage of animals that formed dauers (Neal *et al.* 2013). As expected, wild-type larvae populations formed significantly more dauers in high pheromone conditions (82.2 ± 2.4%) compared to control water plates (4.1 ± 0.8%) (Figure 1A). As controls, we also verified that strains with mutations in TGF-β and insulin-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). Second, we examined dauer formation phenotypes of strains carrying mutations in genes encoding proteins associated with the *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). However, the localization and interaction of *Mutator* proteins in the soma are not well characterized. 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 (Figure 1A). 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 negative control *daf-3*(*mgDf90*), whereas the other strains, including *mut-16(mg461)*, exhibited an intermediate *daf-d* phenotype (Figure 1A). 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 high pheromone conditions (Figure 1A). Interestingly, we found that mutation in another somatic RdRP gene, *rrf-3* (Simmer *et al.* 2002), results in a *daf-d* phenotype similar to the *Mutators*, suggesting that RRF-3 may also interact with *Mutator* proteins in the soma (Figure 1A). These results indicate that a majority of the *Mutator* proteins, which are required for siRNA amplification, are necessary for dauer formation in high pheromone conditions.

Next, we asked which endogenous RNAi pathway contributes to 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* proteins 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 tested whether *nrde-3*(*gg66*) and MAGO12 (carrying mutations in all 12 *wago* genes) mutant strains exhibited *daf* phenotypes in high pheromone conditions. We found that both strains formed dauers similar to wild-type, indicating that *Mutator*-amplified siRNAs critical for dauer formation are not associated with the WAGO RNAi pathways (Figure 1A). Next, we tested whether the nuclear AGO CSR-1 pathway was playing a role in dauer formation using a null mutant, *csr-1(tm182)*, and a hypomorph strain that expresses CSR-1 only in the germline (Claycomb *et al.* 2009). 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, we observed that *csr-1(tm182)* and hypomorph strains exhibited significantly decreased dauer formation levels, similar to the *mut-16*(*pk710*) and *daf-3*(*mgDf90*) strains (Figure 1A). In the germline, 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 a significant *daf-d* phenotype consistent with that seen in the *csr-1(tm182)* and hypomorph strains in high pheromone conditions (Figure 1A). We were unable to test an *ekl-1* mutant strain due to its sterility phenotype. To examine whether MUT-16 and CSR-1 are functioning in the same pathway, we measured dauer formation of a *csr-1(tm182)*; *mut-16(pk710)* double mutant. Interestingly, the double mutant did not show a significant difference in dauer formation compared to *csr-1(tm182)* (Figure 1A) ($P = 0.09$, Student's *t*-test), suggesting that MUT-16 and CSR-1 are functioning in the same pathway. Our results suggest that *Mutator*-amplified siRNAs associating with the CSR-1 pathway are required for dauer formation in high pheromone conditions.

Mutators exhibit stress-specific daf-d phenotypes

Since starvation and high temperature can also trigger dauer formation, we questioned if the *Mutator* and CSR-1 pathway proteins also exhibited a *daf-d* phenotype in different environmental stresses. We subjected newly hatched L1 worms to elevated temperatures (25 and 27°), and measured the proportion of dauer formation (Figure 1B and File S2). At 27°, *mut-2*(*ne298*), *mut-15*(*tm1358*), *mut-16*(*pk710*), *rrf-1*(*pk1417*), and *csr-1* hypomorph strains formed significantly fewer dauers compared to wild-type (Figure 1B). However, except for *mut-16*(*pk710*) and *csr-1* hypomorph, these mutants formed dauers similar to wild-type levels when exposed to a moderately high temperature (25°) (Figure 1B). The *mut-8*(*ne221*) strain, on the other hand, formed significantly more dauers at 25° compared to wild-type. We also noted that the *daf-3*(*mgDf90*) strain exhibited a *daf-d* phenotype at 25°, but a *daf-c* phenotype at 27°, as reported previously (Ailion and Thomas 2000). These results suggest that distinct subsets of *Mutators* are required for dauer formation at different temperatures.

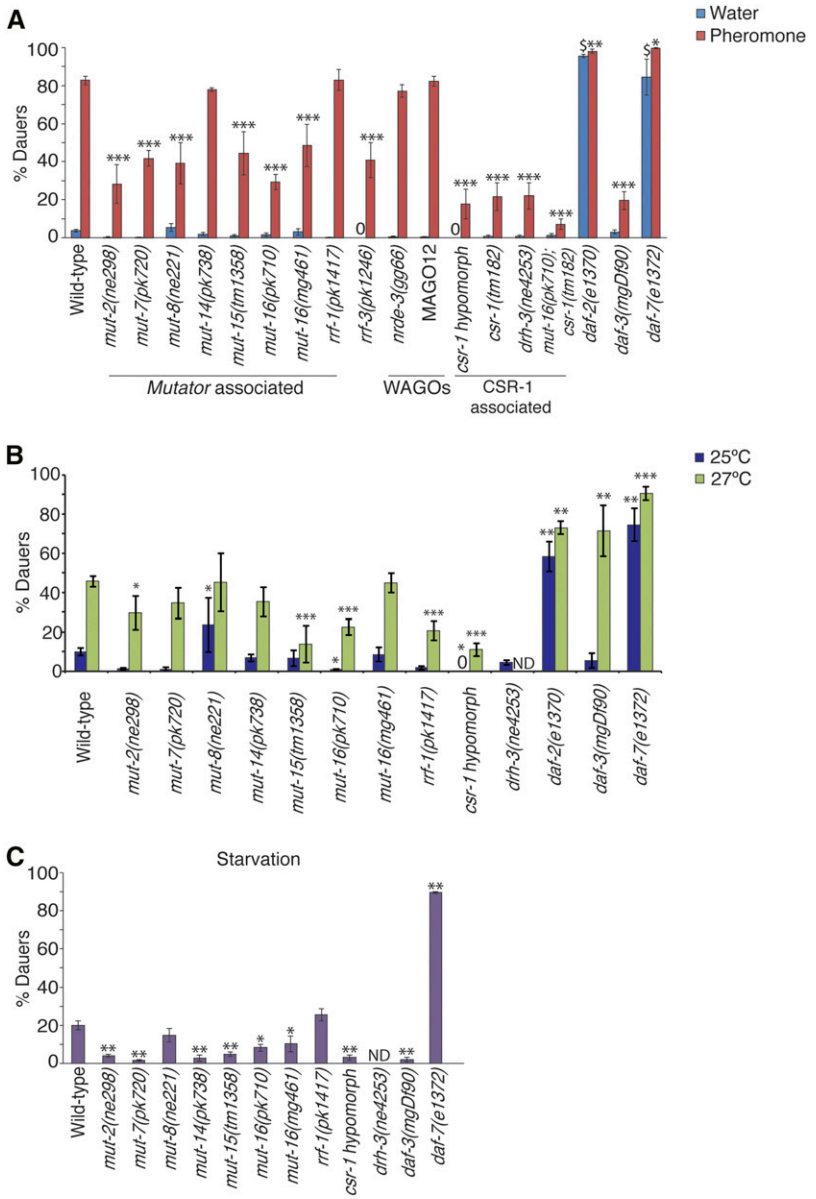

Figure 1 *Mutator* proteins and the CSR-1 AGO pathway are required for dauer formation. (A) Proportion of animals forming dauers in response to high pheromone conditions or water plates is shown. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ compared to wild-type on pheromone plates, $ $P < 0.0005$ compared to wild-type on water plates, one-way ANOVA with LSD *post hoc* test. $N \geq 3$ trials; $n \geq 217$ animals. (B) Proportion of animals forming dauers when cultivated at 25° and 27°. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to wild-type, one-way ANOVA with LSD post hoc test. N ≥ 3 trials; n ≥ 164 animals. (C) 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.

Next, we measured dauer formation in starvation conditions by subjecting newly hatched worms to depleted amounts of *E. coli* (see *Materials and Methods*). We observed that *mut-2*(*ne298*), *mut-7*(*pk720*), *mut-14*(*pk738*), *mut-15*(*tm1358*), *mut-16*(*pk710*), *mut-16*(*mg461*), and *csr-1* hypomorph strains exhibited a *daf-d* phenotype compared to wild-type when exposed to starvation conditions early in development (Figure 1C). However, *mut-8*(*ne221*) and *rrf-1*(*pk1417*) formed dauers not significantly different from wild-type. From these results, we can conclude that a subset of *Mutators* are also required in starvation conditions for dauer formation.

Together, our results indicate that different subsets of RNAi proteins are required for dauer formation in distinct environmental conditions. We have shown that MUT-16 and CSR-1 AGO are required for dauer formation regardless of the environmental stress. However, MUT-2, MUT-7, MUT-8/RDE-2, MUT-14, MUT-15, RRF-1, and RRF-3 are necessary for dauer formation only for specific conditions. These findings indicate that dauer formation phenotypes can be distinct for different environmental stresses, and suggest the possibilities that endogenous RNAi pathways are regulating different subsets of genes, and/or are acting in different cells and tissue types required for dauer formation in high pheromone, starvation, or high temperature conditions.

MUT-16 functions upstream 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 signaling mutants. Prior studies have ordered genes in the TGF-β and insulin 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. We should note here that MUT-16-dependent siRNAs antisense to the coding regions of genes in the insulin signaling and TGF-β dauer formation pathways (including *daf-2*, *daf-7*, *daf-3*, *daf-16*, and *daf-12*) have been detected (Zhang *et al.* 2011), suggesting the possibility that RNAi pathways are regulating dauer formation pathways directly. Only *daf-2* has also been identified as a CSR-1 target, although its mRNA levels do not significantly change in *csr-1* mutants compared to wild-type (Claycomb *et al.* 2009; Cecere *et al.* 2014). Thus, the potential points of interaction between RNAi pathways and dauer formation pathway genes downstream of the sensory detection of adverse conditions, and how that could impact the dauer formation decision, remains unclear.

To perform epistasis analysis, we genetically crossed the *mut-16* (*daf-d*) mutant with *daf-7* (*daf-c*) and *daf-2* (*daf-c*) mutants in the TGF-β and insulin 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 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 (Figure 2). Since MUT-16-amplified siRNAs target *daf-2* and *daf-7*, and the *e1370* allele of *daf-2* is a hypomorph (Gems *et al.* 1998), we cannot conclusively interpret these results without additional experiments that are beyond the scope of this work. However, these results suggest that MUT-16 is not functioning downstream of DAF-2 and DAF-7 for dauer formation. 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 are required in neurons for dauer formation in adverse environmental conditions.

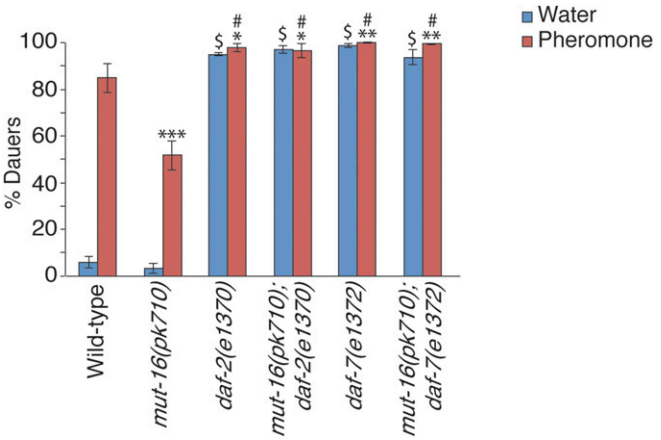

Figure 2 MUT-16 does not function downstream of DAF-7 TGF-β and DAF-2 insulin receptor. Epistasis analysis was performed using dauer formation assays in high pheromone conditions with *mut-16*(*pk710*), *daf-2*(*e1370*), *daf-7*(*e1372*), *mut-16*(*pk710*); *daf-2*(*e1370*), and *mut-16*(*pk710*); *daf-7*(*e1372*) strains. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ compared to wild-type on pheromone plates. # $P < 0.0005$ compared to *mut-16*(*pk710*) on pheromone plates. $ $P < 0.0005$ compared to wild-type on water plates, one-way ANOVA with LSD *post hoc* test. $N \geq 3$ trials; $n \geq 274$ animals. Error bars represent SEM.

Endogenous RNAi pathways are required in neurons for dauer formation

Next, we sought to determine the site of action of RNAi pathways for dauer formation. Based on our results thus far, we hypothesize that *Mutators* and the CSR-1 pathway are functioning in sensory neurons for dauer formation, 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, 1984a; Ren *et al.* 1996; Schackwitz *et al.* 1996; Li *et al.* 2003). To test our hypothesis, we constructed strains expressing a *mut-16*::*gfp* translational fusion driven by a pan-neuronal promoter (*rab-3*) in the *mut-16*(*pk710*) background. We also constructed a *mut-16*::*gfp* rescue transgene that is driven by an intestinal promoter (*ges-1*), since a previous study found that DAF-2 functions in the intestine to regulate dauer formation (Hung *et al.* 2014). Since *mut-16*(*pk710*) exhibited the most significant *daf-d* phenotype in high pheromone conditions, we first subjected the *mut-16* tissue-specific rescue strains to dauer formation assays using high pheromone conditions. As predicted, 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*) (Figure 3A). In contrast, only one transgenic line expressing *ges-1*p::*mut-16*::*gfp* showed a slight but significant increase in dauer formation compared to *mut-16(pk710)*, but remained significantly lower than wild-type (Figure 3A). These results indicate that MUT-16 functions primarily in neurons for dauer formation in high pheromone conditions.

Next, we subjected the *mut-16*::*gfp* tissue-specific rescues to high temperature and starvation dauer formation assays. The pan-neuronal *mut-16*::*gfp* strains exhibited complete, or almost complete, rescue of the *mut-16(pk710) daf-d* phenotype to wild-type levels of dauer formation in high temperature (27°) conditions (Figure 3B). Similarly, the pan-neuronal *mut-16* rescue transgenes completely restored dauer formation in starvation conditions (Figure 3C). In contrast, the intestinal *mut-16* rescue transgenes failed to increase dauer formation levels compared to *mut-16(pk710)*, for both high temperature and starvation conditions (Figure 3, B and C). These results indicate that MUT-16 functions in neurons for dauer formation regardless of experienced environmental stress.

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* animals experience

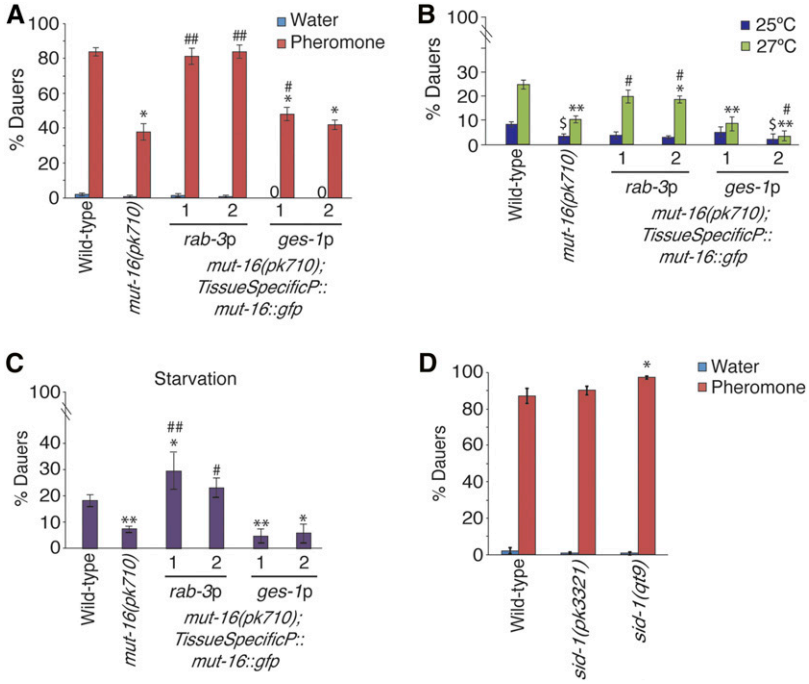

Figure 3 MUT-16 is required in neurons for dauer formation. (A) Proportion of animals forming dauers in response to high pheromone and water plates is shown for pan-neuronal (*rab-3*p) and intestinal (*ges-1*p) *mut-16* rescue strains. * $P < 0.0005$ compared to wild-type on pheromone plates; # $P < 0.05$ and ## $P < 0.0005$ compared to *mut-16*(*pk710*), respectively, on pheromone plates; one-way ANOVA with LSD *post hoc* test. $N \geq 3$ trials; $n \geq 213$ animals. (B) Proportion of animals forming dauers in response to 25 and 27° is shown for pan-neuronal and intestinal *mut-16* rescue strains. * $P < 0.05$, ** $P < 0.0005$ compared to wild-type at 27°; # $P < 0.005$ compared to *mut-16(pk710)* at 27°; $ $P < 0.05$ compared to wild-type at 25°; one-way ANOVA with LSD *post hoc* test. $N = 3$ trials; $n \geq 119$ animals. (C) 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; $n \geq 196$ animals. (D) 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; $n \geq 480$ animals. All error bars represent SEM.

systemic RNAi, a process by which dsRNA can spread throughout cells and tissue types to regulate gene expression (Fire *et al.* 1998; Tabara *et al.* 1998; Timmons and Fire 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, which 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 the *sid-1*(*pk3321*) and *sid-1(qt9)* mutant strains. The *pk3321* allele of *sid-1* resulted in wild-type levels of dauer formation, while the *qt9* allele exhibited a slight but significant increase in dauer formation compared to wild-type (Figure 3D), indicating that systemic RNAi is not required for dauer formation. Together, these results indicate that endogenous RNAi pathways are required in neurons for dauer formation in response to high pheromone and starvation conditions, and, at least in part, for response to high temperature conditions.

RNAi is required in distinct subsets of neurons for dauer formation in different environmental stresses

To further characterize the site of action for the RNAi pathways in the regulation of dauer formation, we examined whether MUT-16 function is required in specific sensory neurons for high pheromone, starvation, and high temperature conditions. Previous work using laser ablation has shown that a subset of amphid sensory neurons (ASI, ADF, and ASG) prevent dauer formation, while others (including ASJ, ASK, and ADL) promote dauer entry (Bargmann and Horvitz 1991; Schackwitz *et al.* 1996). To determine if MUT-16 functions in all, or subsets, of amphid neurons for dauer formation, we generated additional worm strains that express the *mut-16*::*gfp* transgene driven by ASJ, ASI, ADF, or ASG specific promoters, and subjected them to dauer formation assays using high pheromone conditions (Figure 4A). 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 neurons (Figure 4A). Although ASJ, ASI, ADF, and ASG neurons have previously been implicated in dauer formation (Bargman and Horvitz 1991), these results indicate that expression of MUT-16 in either ASI or ASJ neurons is sufficient to rescue the *daf-d* phenotype of *mut-16(pk710)* in high pheromone conditions. Work by others has shown that ASI and ASJ neurons respond to specific pheromone components upstream of DAF-7 TGF-β signaling (Schackwitz *et al.* 1996; McGrath *et al.* 2011; Park *et al.* 2012; Neal *et al.* 2015). Since our epistasis results are consistent with the possibility that RNAi pathway components function upstream of dauer formation pathways, we reasoned that rescue of the *daf-d* phenotypes in high pheromone conditions by expression of MUT-16 in ASI and ASJ neurons may be due to restoration of their ability to sense and/or signal the presence of dauer-inducing pheromone levels. To test this hypothesis, we first verified that these neurons exhibited normal development in *mut-16(pk710)* strains compared to wild-type based on their ability to uptake a lypophilic,

florescent dye (see *Materials and Methods* and Figure S1). Next, we constructed strains that expressed MUT-16 in the ADL neurons, as they have been shown to detect the pheromone component ascr#3 in adults, and the AWA neuron as a negative control, since it has no known role in regulating dauer formation or sensing pheromone components in hermaphrodites (Srinivasan *et al.* 2008; Jang *et al.* 2012). As predicted, we observed that expression of *mut-16*::*gfp* in ADL neurons also resulted in a significant rescue of the *mut-16 daf-d* phenotype, while expression in AWA neurons resulted in no rescue (Figure 4A). Based on these results, we would also predict that ASK-specific expression of *mut-16* would rescue the *mut-16 daf-d* phenotype, since detection of high concentrations of ascr#3 by ASK also promotes dauer formation (Kim *et al.* 2009). ASI, ASJ, and ADL neurons respond to ascr#2 and ascr#3 pheromone components (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, 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.

Since our current model predicts that endogenous RNAi pathways are regulating genes functioning at the sensory detection and/or signaling level of dauer-inducing environmental conditions, we hypothesized that MUT-16 would be required in different neurons for dauer formation in starvation and high temperature compared to high pheromone conditions. The neuronal and molecular mechanisms regulating dauer formation in response to these environmental stresses are not as well characterized as for high pheromone conditions (Hu 2007). Recent work has shown that feeding state is encoded by the calcium/calmodulin-dependent protein kinase I (CMKI) in the AWC and ASI sensory neurons to modulate dauer formation (Neal *et al.* 2015). In addition, AFD neurons have been shown to play a major role in thermotactic behaviors, which are modulated by the AWC chemosensory neurons (Garrity *et al.* 2010); however, their role in the regulation of dauer formation in high temperatures, if any, is unknown. To address whether MUT-16 is required in ASI, ASJ, or ADL neurons for dauer formation in general or specifically in high pheromone conditions, we tested whether the neuron-specific MUT-16 rescue strains exhibited increased dauer formation in high temperature or starvation conditions. At 27°, all of the neuron specific rescues tested formed percentage dauers that were not significantly greater than *mut-16*(*pk710*) levels (Figure 4B). Surprisingly, we observed that some of the neuron-specific MUT-16 rescues exhibited a further decrease in the percentage of dauer formation at 27°. Similarly, we observed no rescue of *mut-16*(*pk710*) *daf-d* phenotype in starvation conditions for the neuron-specific MUT-16 expression strains (Figure 4C), and a significant decrease in dauer formation in one line of ASI-specific MUT-16 expression. These results indicate that MUT-16 is not sufficient in ASJ, ASI, ADF, ASG, ADL, or AWA alone to regulate dauer formation in high temperature or starvation conditions, but may be required in single neurons that remain untested or in subsets of these neurons (Figure 4, B and C). These findings are consistent with a model that the *Mutator* and CSR-1 pathways are required in specific subsets of neurons for dauer formation in different environmental stresses.

CSR-1 promotes expression of G proteins required for dauer formation

Our results thus far predict 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 *C. elegans* neurons, G protein coupled receptors (GPCRs) span the plasma membrane of cilia, and function to detect specific environmental stimuli, including individual pheromone components. When bound to a ligand, the cytoplasmic portion of GPCRs activate their associated G proteins, which function to transduce the sensory signal intracellularly (Koelle 2016). In order to understand the potential molecular mechanism by which endogenous RNAi is required for pheromone sensation, we sought to identify candidate genes involved in sensory signaling that are expressed in ASI, ASJ, and ADL neurons, and exhibit a *daf-d* phenotype when mutated. 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 hypothesize that gene expression of CSR-1 targets required for dauer formation would be downregulated in the *mut-16* and *csr-1* hypomorph strains, resulting in their observed *daf-d* phenotype. We identified three genes encoding G protein subunits, *gpa-1*, *gpa-3*, and *gpc-1*, that are expressed in ASI, ASJ, and ADL and exhibit a *daf-d* phenotype in their respective loss-of-function mutant strains in high pheromone concentrations (Zwaal *et al.* 1997; Lans and Jansen 2007; Kim *et al.* 2009; Hobert *et al.* 2016). 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 cultivated in favorable conditions. We found that expression levels of each of these genes were significantly reduced in either the *csr-1* hypomorph or *mut-16(pk710)* strains, or both, compared to wild-type, indicating that endogenous RNAi promotes expression of these G proteins during the L1 larval stage (Figure 5). In contrast, the expression of the neuropeptide *flp-21* gene, which is also expressed in ASI, ASJ, and ADL neurons, and exhibits no known *daf-d* phenotype when mutated (Li and Kim 2008; Hobert *et al.* 2016), did not exhibit significant changes in mRNA levels in either *mut-16(pk710)* or *csr-1* hypomorph strains (Figure 5). These results are consistent with our hypothesis that the *daf-d* phenotype exhibited by the *csr-1* hypomorph and *mut-16(pk710)* strains in high pheromone

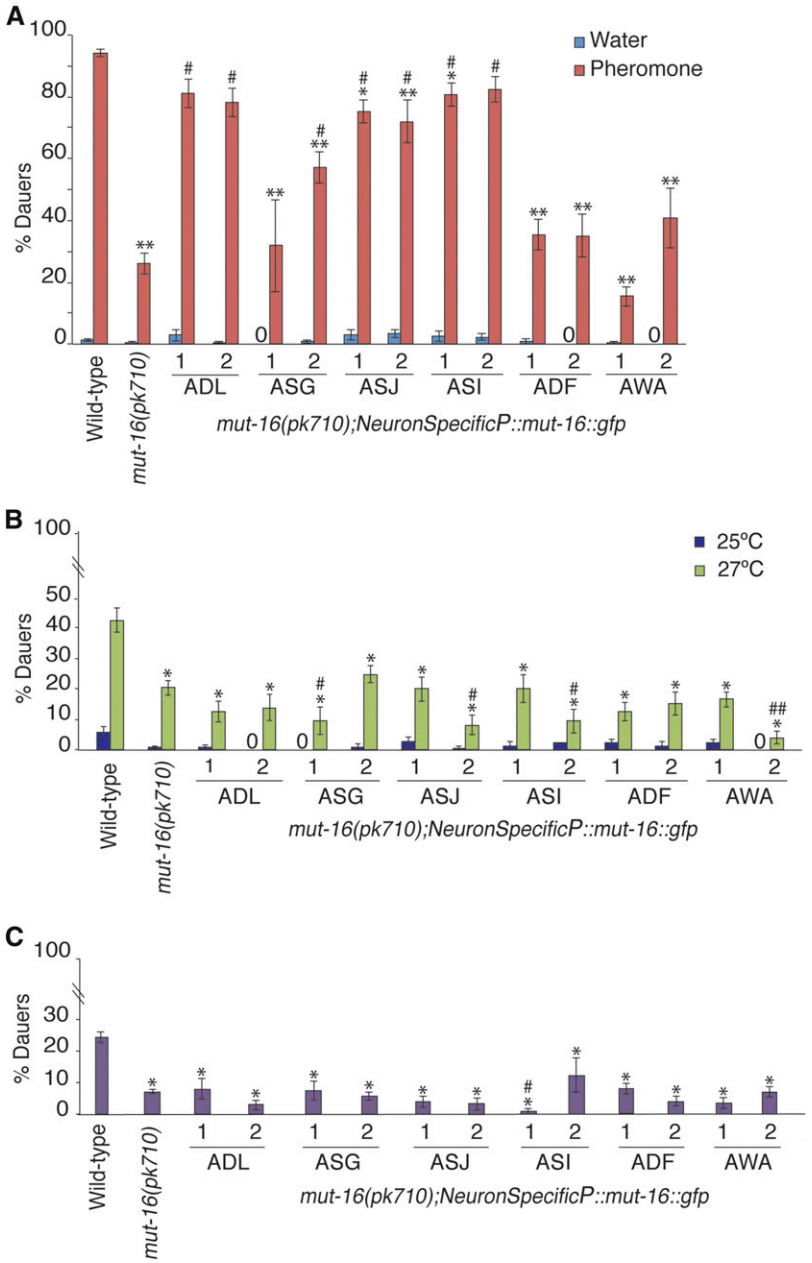

Figure 4 MUT-16 is required in different subsets of neurons for dauer formation in response to different environmental stresses. (A) Proportion of animals forming dauers in response to high pheromone and water control is shown for wild-type, *mut-16*(*pk710*), and neuron-specific rescue strains. $N \geq 3$ trials; $n \geq 173$ 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. (B) Proportion of animals forming dauers in response to 25 and 27° is shown for neuron-specific *mut-16* rescue strains. * $P < 0.0005$ compared to wild-type at 27°; # $P < 0.005$ and ## $P < 0.0005$ compared to *mut-16*(*pk710*) at 27°; one-way ANOVA with LSD post hoc test. $N \geq 3$; $n \geq 120$ at 27° and $N \geq 3$; $n \geq 146$ at 25°. (C) 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. $N \geq 3$; $n \geq 160$. No dauer formation is indicated by "0." All error bars represent SEM. Two independent transgenic lines for each neuron-specific rescue were tested. The dye-filling experiment to verify normal structure of the amphid neurons in *mut-16(pk710)* is shown in Figure S1.

conditions are a result of decreased G protein signaling in neurons that detect high concentrations of ascaroside molecules. Together, our results indicate that endogenous RNAi pathways are required for the basal level of expression of *gpa-1*, *gpa-3*, and *gpc-1* genes essential for sensory signaling early in *C. elegans* development. The reduced expression of these genes in *mut-16(pk710)* and *csr-1* hypomorph animals would result in decreased ability to detect and respond appropriately to environmental cues, and likely contributes to their inability to form dauers in adverse conditions.

Discussion

In this study, we implicate endogenous RNAi as playing a role in the dauer formation behavior in *C. elegans*. We showed that strains carrying mutations in *Mutator* proteins and the nuclear CSR-1 AGO exhibit a *daf-d* phenotype in adverse environmental conditions. In addition, we provide evidence that MUT-16 is required in distinct subsets of neurons for dauer formation in high pheromone concentrations, high temperature, or starvation conditions. In high pheromone conditions, our results indicate that ASI, ASJ, and ADL neurons act as a distributed circuit for promoting dauer formation in high pheromone conditions upstream of the TGF-β and insulin signaling pathways. Furthermore, we provide evidence that suggests that CSR-1 positively regulates expression of a subset of genes that are essential for sensory signaling in neurons that have been shown to detect individual ascarosides. Together, our results suggest a model where *Mutator*-amplified siRNAs acting in the CSR-1 pathway promote expression of

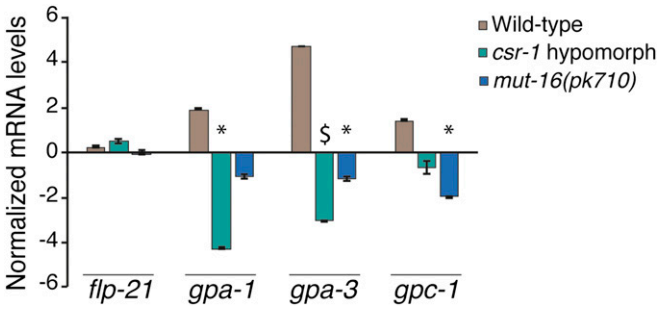

Figure 5 *Mutator* MUT-16 and CSR-1 AGO promote expression of chemosensation genes required for dauer formation. Log2 mRNA levels for *flp-21*, *gpa-1*, *gpa-3*, and *gpc-1* genes in wild-type, *csr-1* hypomorph, and *mut-16(pk710)* strains, normalized to *y45f10d.4*, are shown. * $P < 0.05$ and $^{\$}$ $P < 0.005$ compared to wild-type, one-way ANOVA with LSD *post hoc* test. $N \geq 3$ biological replicates. Error bars represent propagated SD.

genes that are required for the detection and signaling of different environmental stresses in individual sensory neurons, which then signal to promote dauer formation.

RNAi is required in neurons for behavioral adaptation to environment

Since the publication in 1998 reporting that dsRNA has the ability to silence endogenous genes, 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 laboratory 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 downregulation 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 downregulation 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 presented here 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, 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). Since we have shown that expression of MUT-16 in individual ASI, ASJ, or ADL neurons can rescue the *daf-d* phenotype of the *mut-16(pk710)* mutants in high pheromone conditions, we propose a model where *Mutator*-amplified siRNAs associated with CSR-1 promote expression of sensory signaling genes in each of these neurons, which detect high pheromone stress, such as high levels of ascr#5 by ASI neurons (McGrath *et al.* 2011). Detection of high levels of a single ascaroside or ascaroside blends by a single neuron would be sufficient to induce dauer formation, as observed when individual ascarosides added to media can induce dauer formation in wild-type animals (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.

Furthermore, we have shown that pan-neuronal expression of MUT-16 also rescues the *daf-d* phenotype of *mut-16(pk710)* in starvation and high temperature conditions. We have not yet identified the specific neurons where MUT-16 is required for dauer formation during exposure to these stresses; however, our model predicts that it would be in the neurons that detect low food availability or high temperature. 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 the pheromone conditions, 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. Furthermore, the AFD and AWC neurons have been shown to respond to temperature (Garrity *et al.* 2010), which we did not test in our *mut-16* rescue assays. Further experiments are necessary to determine how MUT-16 function is required in low food conditions and high temperatures for dauer formation.

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 rescued the *daf-d* phenotype of *mut-16(pk710)* suggests that a distributed chemosensory circuit functions to detect and promote dauer formation in high pheromone concentrations. 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 observation that dauer formation in high pheromone conditions is distributed among multiple neurons would be expected, given that GPCRs that detect individual ascaroside components are expressed in different neurons: SRBC-64 and SRBC-66 in ASK, and SRG-36, SRG-37, and DAF-37 in ASI (Kim *et al.* 2009; McGrath *et al.* 2011; Park *et al.* 2012). However, what was unexpected was that we rescued

mut-16(pk710) dauer formation defects by expression of MUT-16 in ADL alone, which has not previously been implicated in pheromone detection during early larval stages. 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 unidentified molecules in crude pheromone that are sufficient to 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.

Furthermore, we propose that a distributed chemosensory circuit to detect the presence of dauer-inducing pheromone concentrations provides an adaptive advantage to *C. elegans* animals. The concentrations of the major ascaroside molecules isolated from *C. elegans* populations cultivated at 20° differs from populations cultivated at 25°, indicating that pheromone composition of crude dauer pheromone is dependent upon environmental conditions (Jeong *et al.* 2005; Butcher *et al.* 2007, 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 a crowded area, regardless of other environmental conditions such as temperature.

In this study, we provide evidence that RNAi pathways function early in the dauer formation decision in high pheromone concentrations, by promoting expression of G proteins in neurons that are essential for the detection of individual ascaroside components. 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, and whether the dauer formation triggers may have distinct effects at a molecular level. We have shown previously that postdauer adults that experienced high pheromone conditions are phenotypically distinct from adults that bypassed the dauer stage, and that these effects are partially dependent upon RNAi pathways (Hall *et al.* 2010, 2013). Our data presented here suggest the intriguing possibility that RNAi pathways may mediate distinct effects on gene expression in postdauer animals that experienced different environmental stresses. Since other animals, such as pea aphids, form polyphenisms due to crowding or starvation as well, we predict that this work may have broader implications for the RNAi-mediated regulation of phenotypic plasticity.

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

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