Genetic Analysis of the Neuronal Integrated Stress Response in Developmental Plasticity and Organismal Physiology of *C. elegans*

by
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Abstract

The genetic study of the *C. elegans* dauer developmental decision has served as an experimental paradigm for understanding how environmental cues influence organismal physiology through evolutionarily conserved neuroendocrine signaling mechanisms. My genetic characterization of the previously isolated *daf-28(sa191)* mutant that enters dauer constitutively has revealed cell-nonautonomous roles of conserved stress signaling pathways—the Unfolded Protein Response (UPR) and translational control mediated by eIF2α phosphorylation. While the cell-autonomous functions of these stress-responsive mechanisms in maintaining cellular homeostasis have been examined, their organismal effects on remodeling development and stress responses remain largely unexplored. Chapter II will highlight the hypotheses and approaches that led to identification of the PEK-1/PERK branch of the UPR, functioning in a pair of chemosensory neurons, as a novel regulator of the dauer developmental decision. Chapter III will examine the systemic effects of eIF2α phosphorylation, downstream of PERK/PEK-1 activation, in the sensory nervous system on larval development and stress responses. Specifically, the identification of the *C. elegans* translational regulatory factors that function as molecular determinants of cellular and systemic sensitivity to eIF2α phosphorylation will be described. Subsections of Chapter III and IV will also highlight genes whose functions can modify the organismal effects of the UPR and eIF2α phosphorylation: these genes are involved in modulation of ER proteostasis or function in the dauer neuroendocrine pathways that interact with the UPR or eIF2α phosphorylation. Finally, we proceed to show that alterations in the neuronal eIF2α phosphorylation status may modulate sensory processing to influence diverse physiological outputs, mimicking the effects of starvation or unfavorable microbial environment.

Collectively, results from my study indicate that modulation of the UPR and eIF2α-mediated translational control in the sensory nervous system confers substantial cell-nonautonomous effects on animal physiology. These findings underscore how molecular events underlying cellular homeostasis, which can be perturbed by fluctuating environmental and developmental conditions, may be co-opted to systemically reprogram organismal stress responses in *C. elegans*.

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Chapter I

Genetic Basis of the Dauer Developmental Decision and the Conserved Stress Signaling Mechanisms in the Sensory Nervous System of *C. elegans*

The ability to perceive and respond to adverse environmental challenges is critical for the survival and reproductive fitness of all organisms. In addition to gene expression and biochemical changes at the cellular level, some invertebrates have evolved developmental plasticity termed diapause—a dormant life stage that involves dramatic remodeling of tissues, metabolism and behaviors in response to specific sets of stressors. One striking example is the distinct life stage named *dauerlarven,* the enduring (dauer) larvae, initially identified in parasitic nematodes as an obligate and infective stage in their life cycles (Hotez et al., 1993). The free-living model organism *Caenorhabditis elegans* also enters dauer diapause as an alternative third larval stage in response to unfavorable conditions that include increased population density, food scarcity and high ambient temperature (Cassada and Russell, 1975). The dauer larvae are stress-resistant, long-lived and behaviorally dispersive, enabling *C. elegans* to withstand and circumvent environmental insults (Cassada and Russell, 1975 and Klass and Hirsh, 1976).

Genetic and cellular basis of the *C. elegans* dauer developmental decision

The *C. elegans* dauer developmental decision is a stereotypic and genetically manipulable organism-wide stress response, allowing investigators to dissect the cellular and molecular mechanisms underlying how various external and internal cues affect organismal development. The simple and well-defined nervous system of the worm has

facilitated identification of specific sensory neurons and structures processing the dauer-inducing stimuli and/or modulating the dauer decision (Albert et al., 1981 and Bargmann and Horvitz, 1991). The genetic study of the dauer developmental decision of *C. elegans* has also served as an experimental paradigm for understanding how environmental cues influence organismal physiology through evolutionarily conserved neuroendocrine signaling mechanisms (Riddle et al., 1981). Specifically, insulin, TGFβ and steroid hormone have been shown to play critical roles in regulating the dauer diapause (reviewed in Hu, 2007 and Fielenbach and Antebi, 2008). These conserved neuroendocrine pathways modulating the dauer decision during larval development also regulate physiological stress responses throughout the life cycle, and influence longevity (Kenyon et al., 1993 and Larsen et al., 1995). I am interested in utilizing molecular genetic dissection of the *C. elegans* dauer developmental decision to better understand how perturbations of conserved neuroendocrine signaling mechanisms affect developmental and adaptive stress responses. Of note, while the signal transduction components required for dauer entry have been extensively delineated in the past decades, the molecular events underpinning perception and integration of environmental cues in the context of this organismal stress response have remained largely elusive.

The sensory nervous system and regulation of the systemic stress response

The *C. elegans* sensory nervous system has been shown to mediate diverse physiological outputs such as reproduction and longevity (reviewed in Allen et al., 2015). The amphid sensory organs located in the anterior section of the worm contain neurosecretory cells whose ciliated endings are directly exposed to the external

environment. Importantly, larval development and dauer diapause are controlled by a subset of these amphid neurons, including the ASI chemosensory neuron pair (Bargmann and Horvitz, 1991), which is the focus of my study. The ASI neurons integrate extrinsic and intrinsic cues to regulate the dauer developmental decision via production of TGFβ and insulin-like peptides (Ren et al., 1996, Schackwitz et al., 1996, Li et al., 2003, Cornils et al., 2011 and Hung et al., 2014). Of note, the ASI neuron pair also controls longevity and mediates diet-restriction-induced lifespan extension (Alcedo and Kenyon, 2007 and Bishop and Guarente, 2007). The central roles of the nervous system in regulating organismal stress physiology through neuroendocrine effectors are observed in other invertebrates and higher animals. For instance, a cluster of neurosecretory cells in the *Drosophila* brain produces insulin peptides to coordinate nutrient availability with larval growth and metabolism (Rulifson et al., 2002 and Ikeya et al., 2002). Interoceptive sensory neurons in the mammalian hypothalamus also secrete neuropeptides that regulate energy metabolism and feeding in response to nutrient status signals (Cowley et al., 2003). The well-characterized *C. elegans* chemosensory network and evolutionarily conserved neuroendocrine pathways are suitable for my examination of the adaptive, cell-nonautonomous effects conferred by the central nervous system in response to fluctuating external and internal environmental stimuli.

Homeostatic mechanisms in neuroendocrine cells

The biosynthesis of synaptic outputs and neuroendocrine molecules imposes secretory demands on the endoplasmic reticulum (ER) in the chemosensory neurons. Since imbalance in protein-folding homeostasis causes accumulation of misfolded and

unfolded proteins, a detrimental condition termed ER stress, quality control mechanisms are vital for cellular proteostasis and viability. In this study, I have identified a conserved protein quality control pathway functioning in the sensory neurons to promote the dauer developmental decision: the ER Unfolded Protein Response (UPR) (reviewed in Walter and Ron, 2011). The pivotal roles of the UPR in differentiation and maintenance of specialized secretory cells have been observed in other organisms, with a notable example in olfactory receptor choice commitment in the mammalian sensory neurons (Dalton et al., 2013). My findings will further argue that in addition to its roles in maintaining cellular homeostasis, the UPR also confers cell-nonautonomous, systemic effects such as the dauer diapause. Indeed, the organism-wide consequences of the UPR have been noted in the mammalian central nervous system, where ER stress in the hypothalamic neurons exacerbates systemic glucose metabolism and energy balance (Ozcan et al., 2009 and Williams et al., 2014). Neuronal UPR, in fact, has emerged as an important regulator of systemic proteostasis and metabolism in metazoans (reviewed in Taylor et al., 2014 and Mardones et al., 2015). In *C. elegans*, activation of the neuronal protein quality control mechanisms in the ER, the cytosol and mitochondria coordinates organismal stress resistance, as well as mediates longevity (Taylor and Dillin, 2013, Berendzen et al., 2016 and von Oosten-Hawle and Morimoto, 2014).

Genetic characterization of the *daf-28(sa191)* dauer-constitutive mutant

Genetic studies of dauer formation usually involved characterizations of mutants that fail to form dauers under stressful conditions (Daf-d phenotype) and those that enter diapause constitutively under optimal growth conditions (Daf-c phenotype) (Riddle et al.,

1981). We set out to characterize a Daf-c mutant called *daf-28(sa191)*, initially isolated in the dauer-constitutive genetic screen (Malone and Thomas, 1993). The mutant allele is particularly interesting to me because of its dominant nature, which is unique among previously identified Daf-c mutations, suggesting gain or alteration of function (Malone and Thomas, 1994). Furthermore, the *daf-28(sa191)* mutation triggers constitutive dauer entry largely independently of insulin and TGFβ neuroendocrine signaling mechanisms (Malone et al., 1996). *daf-28* has been shown to encode one of the forty *C. elegans* insulin-like peptides, and the *sa191* mutation results in an arginine-to-cysteine substitution in the DAF-28 peptide (Li et al., 2003).

We have identified a mechanism by which the *daf-28(sa191)* mutation promotes entry into dauer diapause, as described in Chapter II of this study. Our initial hypothesis is that an unpaired cysteine in the DAF-28 insulin is likely to be toxic and disrupts protein-folding homeostasis in the ER, analogous to the mutant insulin genes identified in the Akita diabetes mouse model, as well as in permanent neonatal diabetes mellitus and mutant *INS*-gene induced diabetes of youth (Wang et al., 1999, Stoy et al., 2007 and Liu et al., 2010). As shown in Chapter II, the mutant DAF-28 peptide results in neuronal ER stress and UPR activation. To my surprise, however, instead of resulting in cell death and general dysfunction as in the case of the insulin-dependent diabetes models, the toxic DAF-28 peptide activates the PERK/PEK-1 arm of the UPR in the ASI chemosensory neurons to promote the dauer developmental decision (Chapter II). My study demonstrates a novel role of neuronal UPR signaling in dauer formation, and also underscores cell-nonautonomous, organismal effects of this conserved protein quality control mechanism.

To identify additional genes involved in the dauer developmental decision in response to neuronal UPR activation, I utilized a forward suppressor screen. The rationale for the screen is that mutations in the genes required for the constitutive dauer entry triggered by neuronal ER stress would suppress the Daf-c phenotype. Mutagenized *daf-28(sa191)* animals that failed to enter dauer constitutively were isolated. After the causative mutations were identified, the associated genes/complementation groups were categorized into three distinct classes based on their functions and sites of action, as further described in Chapters III and IV.

Class I: Genes that are involved in the biosynthesis of the toxic DAF-28 insulin peptide and UPR activation in the ASI neurons. Mutations in the genes in this class may only be able to suppress the Daf-c phenotype triggered by neuron-specific misfolded peptides and/or UPR activation. Gene products from this class are likely to be associated with or targeted to the ER and its protein clients. Characterization of Class I genes would allow us to better understand the cellular physiology and stress signaling of the *C. elegans* neurosecretory cells, particularly in the context of organismal development.

Class II: Genes that are involved in cellular and systemic sensitivity to phosphorylation of the translation initiation factor eIF2α, a key molecular event downstream of PERK/PEK-1 activation that results in attenuation of protein synthesis (reviewed in Sonenberg and Hinnebusch, 2009). Gene products from this class are likely to play important roles in translational control, specifically at the translation initiation steps. Characterization of Class II genes would illuminate the molecular basis of translation inhibition by the conserved eIF2α phosphorylation mechanism, specifically in the context of *C. elegans* larval development and organism-wide stress responses.

Class III: Genes that are involved in mediating the dauer developmental decision through neuroendocrine effectors. Gene products from this class are likely to play key roles in signal transduction in tissues that produce or receive endocrine molecules functioning to coordinate the morphological, metabolic and behavioral remodeling processes associated with the dauer commitment. We note that because the Daf-c phenotype of the *daf-28(sa191)* mutant is partially dependent on the FOXO transcription factor downstream of the insulin receptor (Malone et al., 1996), some of the genes in this class may function as part of or to modulate the insulin signaling pathway. Class I and Class II genes, on the other hand, operate independently of the insulin signaling mechanism, as well as other previously characterized dauer signaling pathways.

Perspectives of the study

My characterization of the *daf-28(sa191)* mutant has revealed the cell-nonautonomous roles of conserved stress signaling mechanisms—the UPR and translational control—in the *C. elegans* sensory nervous system. Chapter II of this study will discuss the approaches and findings that have led to identification of the PERK/PEK-1 branch of the UPR, functioning in a pair of chemosensory neurons, as a regulator of the dauer developmental decision. Chapter III will highlight forward-genetics-derived mutant analysis that has helped illuminate the systemic roles of neuronal eIF2α phosphorylation and conserved translational regulatory factors in both larval development and stress responses throughout life. Of note, key observations in Chapter III indicate that modulation of the neuronal eIF2α phosphorylation status can alter sensory integration of physiological dauer-inducing stimuli such as food sources. A subsection of Chapter III

will also highlight genes involved in maintenance of ER homeostasis and biosynthesis of neuropeptides in the chemosensory neurons. Chapter IV will describe genes that participate in conserved stress signal transduction mechanisms that have not been previously characterized in the context of the *C. elegans* dauer diapause. Interestingly, some of these genes also function to modulate the insulin pathway, suggesting crosstalk of various stress-responsive mechanisms. Taken together, results from my study suggest that modulation of stress signaling pathways in the sensory nervous system dramatically remodels animal physiology. These findings underscore how molecular events underlying cellular homeostasis, which can be perturbed by fluctuating environmental and developmental milieu, may be co-opted to systemically reprogram organismal stress responses in *C. elegans*.

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Chapter II

The Unfolded Protein Response in a Pair of Sensory Neurons Promotes Entry of *C. elegans* into Dauer Diapause

This chapter is adapted from our manuscript published in *Current Biology* in December 2013. The numbered references are specific to this chapter and listed towards the end.

Abstract

In response to unfavorable environmental conditions such as starvation, crowding, and elevated temperature, *Caenorhabditis elegans* larvae enter an alternative developmental stage known as dauer [1], which is characterized by adaptive changes in stress resistance and metabolism [2, 3]. The genetic dissection of the molecular mechanisms of the *C. elegans* dauer developmental decision has defined evolutionarily conserved signaling pathways of organismal neuroendocrine physiology [2–4]. Here, we have identified a mechanism by which a dominant mutation in a neuronal insulin gene, *daf-28(sa191)* [5–7], causes constitutive entry into dauer diapause. We demonstrate that expression of the mutant DAF-28 insulin peptide results in endoplasmic reticulum (ER) stress in the ASI pair of chemosensory neurons. The neuronal ER stress does not compromise cellular survival but activates PEK-1, the *C. elegans* ortholog of the mammalian eIF2α kinase PERK, which in turn phosphorylates Ser49 of eIF2α, specifically in the ASI neuron pair, to promote entry into dauer diapause. Our data

establish a novel role for ER stress and the unfolded protein response (UPR) in promoting entry into dauer diapause and suggest that, in addition to cell-autonomous activities in the maintenance of ER homeostasis, the UPR may act in a non-cell-autonomous manner to promote organismal adaptation to stress during larval development.

Results

The daf-28(sa191) Mutation Causes ER Stress in the ASI Chemosensory Neuron Pair

The *daf-28(sa191)* mutation results in constitutive entry into dauer diapause, partially independent of previously identified insulin and TGF-β-signaling pathways ([6]; see also Table S1), but the mechanism is not well understood [5, 6, 7]. Molecular characterization of the *daf-28(sa191)* mutant revealed that the *sa191* mutation results in an arginine-to-cysteine substitution at the predicted proteolytic cleavage site of the DAF-28 insulin peptide [7]. Genetic studies established that the *daf-28(sa191)* allele is semidominant and results in altered insulin/DAF-28 activity [5, 7]. Our characterization of two putative null alleles of *daf-28*, *daf-28(tm2308)* [8] and *daf-28(gk411072)*, alongside *daf-28(sa191)* corroborated these data (Figure 1A; Table S2).

In considering the gain-of-function nature of the *sa191* allele, we hypothesized that the *sa191* mutation might cause the production of a toxic insulin peptide that could disrupt protein folding homeostasis in the endoplasmic reticulum (ER). Such a mechanism would be reminiscent of the Akita mouse model of insulin-dependent diabetes, in which a dominant mutation in an insulin gene causes ER stress in pancreatic beta cells [11]. Expression of the *daf-28* gene was previously shown to be restricted to the ASI and ASJ chemosensory neurons [7]. To determine whether the *sa191* mutant DAF-

28 insulin peptide disrupts ER homeostasis, we utilized a fluorescent reporter transgene in which GFP is under the control of the promoter of *hsp-4*, a *C. elegans* ortholog of the ER chaperone BiP. Expression of *hsp-4p::GFP(zcIs4)* is induced by ER stress in diverse tissues [12]. We observed induction of GFP expression specifically in the ASI neuron pair in the *daf-28(sa191)* animals, but not in the ASI neuron pairs of wild-type or *daf-28* loss-of-function mutant animals (Figure 1B). No *hsp-4p::GFP* expression was observed in the ASJ neuron pair of *daf-28(sa191)* animals.

Single-molecule fluorescence in situ hybridization (smFISH) [13] confirmed upregulation of endogenous *hsp-4* mRNA in the ASI neuron pair in the *daf-28(sa191)* mutant (Figure 1C). The highly sensitive smFISH method also revealed expression of *hsp-4* mRNA in cells other than the ASI neuron pair in the *daf-28(sa191)* mutant (Figures 1C; Figures S1A and S1B), but we observed that these cells were not the ASJ neuron pair (Figure S1A). We confirmed that the non-ASI, non-ASJ neuron expression of *hsp-4* mRNA in the *daf-28(sa191)* mutant overlapped with additional cells expressing *daf-28* as determined by smFISH analysis of *daf-28* mRNA expression (Figures S1A and S1B).

We also observed *hsp-4p::GFP* expression in the ASI neurons in the *daf-28(sa191)/+* heterozygote, consistent with the dominant nature of the *sa191* allele (Figure S1C). ER stress induces *hsp-4* expression in an IRE-1-XBP-1-dependent manner [12]. GFP expression in the ASI neuron pair of the *daf-28(sa191)* mutant was dependent on XBP-1, demonstrating that the observed upregulation of *hsp-4* expression was a consequence of ER stress and unfolded protein response (UPR) activation (Figure 1B). Further evidence that *daf-28(sa191)* mutant insulin expression is sufficient to trigger ER stress and UPR activation in a cell-autonomous manner was obtained from ectopic

overexpression of *daf-28* in the intestine. We observed that intestinal expression of the *sa191* mutant DAF-28 peptide, but not wild-type DAF-28, resulted in the induction of *hsp-4p::GFP* expression (Figure S1D). The mitochondrial UPR and the cytosolic heat-shock response, as well as genes mediating oxidative stress response and autophagy, were not activated in the ASI neurons of the *daf-28(sa191)* mutant (data not shown), establishing that expression of the mutant DAF-28 insulin peptide specifically causes disruption of ER homeostasis in the ASI neuron pair.

The ASI Neuron Pair Remains Intact in the daf-28 (sa191) Mutant

The ASI neuron pair has been shown to play an important role in the dauer decision [14], and thus we considered whether neuronal ER stress promotes the dauer decision by compromising the survival of the ASI neuron pair. Three lines of evidence argue against such a mechanism. First, while laser ablation of the ASI neurons, in combination with a few other chemosensory neurons, results in constitutive entry into dauer diapause, dauer entry caused by chemosensory neuron ablation is dependent on DAF-3/SMAD [14], whereas the constitutive dauer entry phenotype of the *daf-28 (sa191)* mutant is independent of DAF-3 ([6] and Table S3). Second, the ASI neurons of the *sa191* animals appeared morphologically intact (Figure 1B; Figure S2C) and expressed the *daf-28p::GFP* transcriptional reporter (Figure S2A). Third, the ASI neuron pair of the *daf-28(sa191)* mutant are functional for the secretion of the transgenic translational fusion DAF-28::GFP (Figure S2B). These data strongly suggest that neuronal ER stress in the *daf-28(sa191)* mutant promotes entry into dauer diapause without compromising the survival of the ASI neuron pair.

Activation of the PERK/PEK-1 Branch of the UPR in the ASI Neuron Pair Promotes Entry into Dauer Diapause

Having determined that neuronal ER stress does not cause cell death in the *daf-28(sa191)* mutant, we sought to define the mechanism by which the ASI-specific ER stress promotes dauer entry. We hypothesized that the dauer developmental decision might be promoted by activation of the UPR, which in metazoans is comprised of three principal branches, mediated by IRE-1-XBP-1, PERK/PEK-1, and ATF-6 [15, 16, 17]. We found that loss-of-function mutations in the genes encoding IRE-1, XBP-1, and ATF-6 did not affect the constitutive dauer entry phenotype of the *daf-28(sa191)* mutant, indicating that dauer entry is not triggered by the activation of IRE-1-XBP-1 and ATF-6 in response to ER stress (Figure 2A). Of note, at a lower temperature where the *daf-28(sa191)* mutation is less penetrant, mutations in *xbp-1* and *atf-6* that disrupt ER homeostasis enhanced dauer formation in the *daf-28(sa191)* background (Figure S1E), consistent with our hypothesis that the *daf-28(sa191)* mutation causes dysregulation of ER homeostasis to promote the dauer decision.

In contrast, loss-of-function mutations in *pek-1*, which encodes the conserved ER stress-responsive eIF2α kinase PERK/PEK-1 [18], suppressed the constitutive dauer entry phenotype of *daf-28(sa191)* animals (Figure 2B), suggesting that the constitutive dauer entry phenotype of the *daf-28(sa191)* mutant is dependent on PEK-1. Mutation of *gcn-2*, encoding an ER stress-independent eIF2α kinase, had no effect on dauer entry in the *daf-28(sa191)* mutant (Figure S3A). Mutation of *pek-1* had no effect on dauer formation in wild-type animals or in dauer-constitutive mutants carrying mutations in

genes encoding components of insulin and TGF-β-signaling pathways (Figure 2C), demonstrating that PEK-1 activity is required for dauer entry specifically in response to neuronal ER stress in the *daf-28(sa191)* mutant, and that insulin and TGF-β-signaling mechanisms for dauer entry are not dependent on PEK-1. Because we observed ER stress specifically in the ASI neuron pair of *daf-28(sa191)* animals (Figure 1), we anticipated that the corresponding activation of PEK-1 in the ASI neuron pair would be sufficient to promote entry into dauer diapause. Consistent with this expectation, ASI-specific expression of *pek-1* was able to restore the constitutive dauer entry phenotype of the *daf-28(sa191);pek-1(ok275)* mutant, demonstrating that PEK-1 activation by ER stress in the ASI neuron pair is sufficient to promote entry into dauer diapause (Figure 2D; Figure S3B).

The Dauer Developmental Decision Is Dependent on the Phosphorylation State of Ser49 of eIF2α in the ASI Neuron Pair

To further define the mechanism by which ASI-specific UPR activation promotes the dauer developmental decision, we asked whether ASI-specific eIF2α phosphorylation downstream of PEK-1 activation is required for the *daf-28(sa191)* mutant to constitutively enter dauer diapause. Ser49 is the conserved phosphorylation site of *C. elegans* eIF2α [19]. We overexpressed an unphosphorylatable version of *eIF2α(S49A)* specifically in the ASI neurons of the *daf-28(sa191)* mutant and observed partial suppression of the constitutive dauer entry phenotype (Figure 3A). We then asked whether ASI-specific eIF2α phosphorylation could functionally substitute for PEK-1 activation to promote dauer entry in response to neuronal ER stress. We introduced the

ASI-specific phosphomimetic *eIF2α(S49D)* transgene into the *daf-28(sa191);pek-1(ok275)* mutant and found that this transgene substantially restored the constitutive dauer entry phenotype of the *daf-28(sa191)* mutant that was suppressed by *pek-1(ok275)* (Figure 3B). The ASI neurons appeared intact in animals where the cell-specific *eIF2α(S49D)* transgene was expressed in the *daf-28(sa191);pek-1(ok275)* background (Figure S2C). Importantly, overexpression of the wild-type version of *eIF2α* in the ASI neuron pair did not influence the dauer developmental decision in response to neuronal ER stress (Table S3). To further establish that phosphorylation of Ser49 of eIF2α in the ASI neuron pair is the key event downstream of ER stress induced by *daf-28(sa191)* to promote dauer entry, we introduced the ASI-specific phosphomimetic *eIF2α(S49D)* transgene into the *daf-28(tm2308)* null mutant, and we observed that this transgene was sufficient to confer the constitutive dauer entry phenotype (Figure S3C). These data suggest that PEK-1 promotes dauer diapause through the phosphorylation of Ser49 of eIF2α in the ASI neuron pair.

Discussion

The genetic study of the dauer diapause in *C. elegans* has served as an experimental paradigm for understanding how environmental cues influence organismal physiology through conserved neuroendocrine signaling mechanisms [1, 2, 3, 4]. Our data demonstrate that ER stress in the ASI neuron pair, arising from the *sa191* mutant DAF-28 insulin peptide, triggers activation of the conserved eIF2α kinase PEK-1 to promote entry into dauer diapause (Figure 3C). These data establish a previously uncharacterized mechanism promoting entry into dauer diapause in *C. elegans* and

moreover suggest that PEK-1 phosphorylation of eIF2α not only functions to maintain ER homeostasis in the ASI neuron pair but can have non-cell-autonomous effects on larval development and organismal physiology.

Mutations in insulin genes have been shown to result in disruption in proinsulin processing, leading to ER stress and activation of the UPR [9, 11]. In the Akita mouse model [11], dominant mutations in an insulin gene impair proinsulin folding and result in chronic UPR activation that leads to dysfunctional pancreatic beta cells. Such effects of ER stress on pancreatic beta cells are cell autonomous, causing compromised cellular function and subsequent cell death in response to ER stress [16]. Because the ASI neuron pair plays a central neuroendocrine role in the regulation of *C. elegans* physiology, mediating food sensing, and dauer formation and longevity [7, 10, 14, 20, 21, 22, 23], we initially considered that death and/or dysfunction of the stressed ASI neurons might underlie the observed *daf-28(sa191)* phenotype. However, our genetic analysis (Figure S2; Table S3) suggests that the *daf-28(sa191)* mutation promotes dauer entry through a mechanism distinct from that observed from ASI ablation. Moreover, the suppression of the *daf-28(sa191)* constitutive dauer entry phenotype by the *pek-1* mutation (Figure 2) strongly suggests that the activation of PEK-1 and phosphorylation of eIF2α, not the toxic consequences of neuronal ER stress on cell survival, regulate the dauer entry decision. Our data suggest that neuronal ER stress in the *daf-28(sa191)* mutant activates UPR signaling and specifically PEK-1 to phosphorylate Ser49 of eIF2α in the ASI pair of neurons, which has organism-wide effects on larval development without causing death of the ASI neuron pair.

The *sa191* mutant DAF-28 peptide triggers ER stress in the ASI neurons, but not

in the ASJ neurons (Figure 1; Figures S1A and S1B). The lack of detectable UPR activation in the ASJ neuron pair (Figures S1A and S1B) may be a consequence of lower *daf-28* expression levels compared with expression in the ASI neuron pair, or differences in threshold of the ASI and ASJ neuron pairs in response to perturbation in ER homeostasis. The ASI neurons secrete a broad array of neuropeptides [7, 10, 20, 21] and thus may be more susceptible to disruptions that affect their secretory functions. We speculate that just as we have observed that infection with pathogenic bacteria and activation of innate immunity in the intestine activate the UPR [24], similarly adverse environmental conditions may trigger neuroendocrine responses in the ASI neuron pair that may induce ER stress and UPR activation. Alternatively, or in addition, by analogy to the induction of ER stress by metabolic dysregulation in mammals [25], fluctuations in nutrients and metabolites may disrupt ER homeostasis of ASI sensory neurons that regulate *C. elegans* organismal physiology [7, 10, 14, 20, 21, 22, 23].

The activation of the mitochondrial UPR, ER UPR, and the heat-shock response in specific tissues of *C. elegans* has been shown to alter stress response pathways of distal cells through the secretion of postulated "mitokines," a secreted ER stress signal, and a transcription factor FoxA-dependent transcellular chaperone signaling mechanism, respectively [26, 27, 28]. Because the ASI neuron pair does not directly innervate the downstream tissues that are remodeled during the dauer diapause, we speculate that the disruption of ER homeostasis in a pair of neurons may promote entry into dauer diapause through the secretion of neuroendocrine signals that are produced in the ASI neuron pair in response to eIF2α phosphorylation by PEK-1. Previous studies have established that DAF-16/FoxO, downstream of insulin signaling, is required for the constitutive dauer

entry of the *daf-28(sa191)* mutant ([6]; Table S1). The partial suppression of the *daf-28(sa191)* dauer-constitutive phenotype by *daf-16* may be due in part to the reduction of insulin signaling caused by loss of functional DAF-28, which is consistent with the observation that the *daf-28(tm2308)* null mutation confers a weak constitutive entry into dauer phenotype that is suppressed by *daf-16* mutation [8]. PEK-1 phosphorylation of eIF2α in the ASI neuron pair may also activate DAF-16-dependent pathways, for example through the altered translation of insulin peptides that mediate DAF-16 activity and the dauer developmental decision.

Roles for the mammalian UPR in plasma cell differentiation [29] and the recent report of olfactory receptor neuron fate commitment [30] have underscored the function of the UPR in not only maintaining cellular ER homeostasis but also triggering developmental cell fate programs. Furthermore, ER stress in liver cells has been shown to influence diverse physiological outputs such as glucose metabolism [31] and iron homeostasis [32]. Our data suggest not only that the UPR functions to restore and maintain cellular ER homeostasis in the two ASI neurons but also that UPR activation in the ASI neuron pair can promote an organismal stress adaptation, namely entry into dauer diapause, during larval development. Evolutionary conservation of the mechanisms described in our studies would implicate a pivotal role for ER homeostasis and UPR signaling in how cellular stress and translational status are perceived and communicated in the physiology and development of more complex animals.

Experimental procedures

Caenorhabditis elegans strains

C. elegans strains were maintained as previously described [33]. The following strains were used: N2 Bristol wild-type strain, JT191 *daf-28(sa191)*, ZD699 *daf-28(tm2308)*, ZD940 *daf-28(gk411072)*, SJ4005 *zcIs4[hsp-4p::GFP]*, ZD702 *daf-28(sa191);zcIs4[hsp-4p::GFP]*, ZD759 *daf-28(tm2308);zcIs4[hsp-4p::GFP]*, ZD801 *xbp-1(tm2482);daf-28(sa191);zcIs4[hsp-4p::GFP]*, FK181 *ksIs2[daf-7p::GFP]*, ZD1064 *daf-28(sa191);ksIs2[daf-7p::GFP]*, ZD704 *xbp-1(tm2482);daf-28(sa191)*, ZD937 *ire-1(v33);daf-28(sa191)*, ZD846 *daf-28(sa191);atf-6(ok551)*, ZD823 *daf-28(sa191);pek-1(ok275)*, ZD824 *daf-28(sa191);pek-1(tm629)*, MC366 *pek-1(ok275)*, DR1572 *daf-2(e1368)*, ZD915 *daf-2(e1368);pek-1(ok275)*, ZD715 *daf-7(ok3125)*, ZD916 *daf-7(ok3125);pek-1(ok275)*, ZD842-844 *daf-28(sa191);pek-1(ok27 5);qdEx48-50[gpa-4p::pek-1::unc-54 3'UTR]*, ZD928-929 and 932 *daf-28(sa191);qdEx51-53[gpa-4p::eIF2α(S49A)::unc-54 3'UTR]*, ZD951-953 *daf-28(sa191);pek-1(ok275);qdEx54-56[gpa-4p::eIF2α(S49D)::unc-54 3'UTR]*, GR1455 *mgIs40[daf-28p::GFP]*, ZD803 *daf-28(sa191);mgIs40[daf-28p::GFP]*, OE3010 *ofEx4[trx-1p::TRX-1::GFP]*, ZD1065 *daf-28(sa191);ofEx4[trx-1p::TRX-1::GFP]*, ZD747 *svIs69[daf-28p::DAF-28::GFP]*, ZD805 *daf-28(sa191);svIs69[daf-28p::DAF-28::GFP]*, ZD1066-1068 *zcIs4[hsp-4p::GFP]; qdEx80-82[ges-1p::daf-28(+)::unc-54 3'UTR]*, ZD1069-1071 *zcIs4[hsp-4p::GFP]; qdEx80-82[ges-1p::daf-28(sa191)::unc-54 3'UTR]* ZD815 *gcn-2(ok871);daf-28(sa191)*, ZD954-956 *daf-28(sa191);pek-1(ok275);qdEx57-59[str-3p::pek-1::unc-54 3'UTR]*, ZD933, 1021 and 1023 *daf-28(tm2308);qdEx74-76[gpa-4p::eIF2α(S49D)::unc-54 3'UTR]*, ZD967-969 *daf-28(sa191);qdEx60-62[gpa-4p::eIF2α(WT)::unc-54 3'UTR]*, ZD 995-997 *daf-28(sa191);pek-1(ok275);qdEx71-73[gpa-4p::eIF2α(WT)::unc-54 3'UTR]*,

GR1311 *daf-3(mgDf90)*, ZD822 *daf-28(sa191);daf-3(mgDf90)*, ZD939 *daf-16(mgDf47); daf-28(sa191);daf-3(mgDf90)* and ZD941 *daf-16(mgDf47);daf-3(mgDf90)*.

The strain carrying *daf-28(tm2308)* allele was kindly provided by G. Ruvkun. The strain carrying *pek-1(ok275)* was kindly provided by M. Crowder. The strain carrying *svIs69* was kindly provided by S. Tuck. Unless otherwise noted, the ZD strains have been outcrossed at least three times. ZD940 was outcrossed twice. Strains carrying the *pek-1(tm629)* and *atf-6(ok551)* alleles were outcrossed once to the *daf-28(sa191)* strain. In addition to using two independent alleles of *pek-1*, the transheterozygote between the *pek-1(ok275)* and *pek-1(tm629)* alleles was generated in the *sa191* background to confirm the suppression of the Daf-c phenotype by *pek-1* loss-of-function mutations. Double and triple mutants were generated and genotyped using standard methods (relying on PCR or visible phenotypes, when possible). Experiments involving transheterozygotes were performed by crossing the two indicated strains. The mated P_0 hermaphrodites were allowed to lay eggs under the same condition as the original strains, and the F_1 cross progeny was confirmed and scored as L4 or dauer. The *daf-28(sa191)/daf-28(gk411072)* and *daf-28(tm2308)/daf-28(gk411072)* heterozygotes were derived from crosses involving the strain VC30082, carrying the *daf-28(gk411072)* allele.

Constructs and generation of transgenic lines

Transgenic animals, carrying extrachromosomal arrays, were generated using standard microinjection methods [34]. Unless otherwise noted, injection mixture includes an indicated PCR fusion construct (injected at 15 ng/µl) and the *ofm-1p::GFP* co-injection marker (pQZ22, injected at 50 ng/µl), kindly provided by J. Alcedo. PCR fusion

was performed as previously described [35]. The final heterologous constructs and their precursors were pooled from at least eight independent PCRs to minimize misincorporation rates, using high-fidelity DNA polymerase. The expected sizes of the constructs were confirmed by agarose gel electrophoresis.

The promoter of the *gpa-4* gene was used as a cell-specific promoter to express transgenes. A second, independent ASI-specific promoter, *str-3p*, was also used to corroborate our results.

The *gpa-4p::pek-1::unc-54 3'UTR* construct includes 2.9 kb of *gpa-4* promoter, 4.2 kb of *pek-1* genomic region (including short introns) and 0.7 kb of *unc-54* 3'UTR. The *gpa-4* promoter region was amplified by using 5′ primer 5′-ATCACACCGTCGTGAGCTA-3′ and 3′ primer 5′-CTATATAATACACACTCATTG TTGAAAAGTGTTCACAAAATG-3′ (contains an overhang complementary to the *pek-1* genomic region for subsequent PCR fusion). The *pek-1* genomic region was amplified by using 5′ primer 5′-ATGAGTGTGTATTATATAG-3′ and 3′ primer 5′-AGGCACGGGCGCGAGATGTTATTGGAGAAATTTATGAG-3′ (contains an overhang complementary to the *unc-54* 3'UTR region for subsequent PCR fusion). The *unc-54* 3'UTR region was amplified by using 5′ primer 5′-CATCTCGCGCCCGTGCCT-3′ and 3′ primer 5′-AAGGGCCCGTACGG CCGACTAGTAGG-3′. Nested primers were used in subsequent PCR fusion, as previously described. The *str-3p::pek-1::unc-54 3'UTR* construct includes 2.9 kb of *str-3* promoter, 4.2 kb of *pek-1* genomic region (including short introns) and 0.7 kb of *unc-54* 3'UTR. The *str-3* promoter region was amplified by using 5′primer 5′-TTCAGAAGGCAGATGCAAAA-3′ and 3′ primer 5′-TAAAACTATATAATACACACTCATGTTCCTTTTGAAATTGAGGCAGT-3′

(contains an overhang complementary to the *pek-1* genomic region for subsequent PCR fusion). The *str-3p::pek-1::unc-54 3'UTR* construct was injected at 40 ng/μl.

The *gpa-4p::eIF2α::unc-54 3'UTR* construct includes 2.9 kb of *gpa-4* promoter, 1.1 kb of *eIF2α* cDNA (wild-type, phosphomimetic and unphosphorylatable versions kindly provided by S.Takagi) and 0.7 kb of *unc-54* 3'UTR. The *gpa-4* promoter and *unc-54* 3'UTR regions were amplified as described above. The 3' primers used to amplify *gpa-4p* contain an overhang complementary to the *eIF2α* cDNA region for subsequent PCR fusion. An indicated version of *eIF2α* was amplified by using 5' primer 5'-ATGAAATGCCGTTTCTACGAG-3' and 3' primer 5'-AGGCACGGGCGCGAGA TGTT AATCATCCTCCTCATCACTGT-3' (contains an overhang complementary to the *unc-54* 3'UTR region for subsequent PCR fusion). Nested primers were used in subsequent PCR fusion, as previously described.

The *ges-1p::daf-28::unc-54 3'UTR* construct includes 1.5 kb of *ges-1* promoter, 0.7 kb of *daf-28* genomic region (including an intron) and 0.7 kb of *unc-54* 3'UTR. The *ges-1* promoter region was amplified by using 5' primer 5'-CTTCGGGCGCTACCAATAAG-3' and 3' primer 5'-GATGGCGATGAGCTTGCAGT TCATCTGAATTCAAAGATAAGATATGT-3' (contains an overhang complementary to the *daf-28* genomic region for subsequent PCR fusion). The *daf-28* genomic region (wild-type and *sa191* versions) was amplified by using 5' primer 5'-TCTCCTCTCAACAACTATCTCAACA-3' and 3' primer 5'-AGTCAGAGGCACGGG CGCGAGATGTTAAAGAAGCAAACGTGGGCAA-3' (contains an overhang complementary to the *unc-54* 3'UTR region for subsequent PCR fusion). Nested primers

were used in subsequent PCR fusion, as previously described. The *ges-1p::daf-28::unc-54 3'UTR* construct was injected at 50 ng/μl.

Dauer formation assay

Six to eight gravid animals were picked to individual well-seeded 6-cm NGM plates, allowed to lay eggs at the assay temperature for three to six hours, and removed. Live *E. Coli* strain OP50 was used as a food source. For the assays conducted at 25°C, dauer and L4 larvae were scored at 48 hours after the egg-lay midpoint, as at this time point all the animals have passed the pre-dauer stages and, for the dauer-constitutive mutants that exit dauer, the dauer larvae have not resumed reproductive development. Dauers were discriminated from non-dauers based on radial shrinkage of the body, the absence of pharyngeal pumping, and an overall dark appearance [1]. Resistance to 1% detergent can be used to definitively confirm the dauer identity. To minimize variation in environmental conditions that could influence dauer formation, the same position in the incubator was used in all the experiments for temperature consistency, and the population density on each plate was controlled by the number of gravid adults laying eggs and the duration of egg-lay.

For dauer assays involving transgenic strains, gravid extrachromosomal-array-carrying hermaphrodites were used for egg lay, and both non-transgenic and transgenic offspring were scored as dauer or L4. Fractions of dauers were then derived for both non-transgenic and transgenic cohorts. Paired Student's t-test analyses between transgenic and non-transgenic dauer fractions from each trial were conducted for all lines. At least three independent lines were tested for each transgenic construct.

Microscopy

Animals were mounted with 10mM sodium azide onto slides with a 2% agarose pad. The slides were viewed using an AxioImager Z1 fluorescence microscope (Zeiss) primarily with a x40/1.3 (oil) objective. The fluorescence signals were recorded by a CCD camera (AxioCam), using constant exposure time without saturation. The images were captured and processed using the AxioVision image processor software. Lipophilic dye DiI was used to identify amphid neurons.

DiI stock was diluted in M9 to the final concentration of 10 μg/ml. Worms raised at 22.5 ℃ or 25℃ were transferred to the solution and incubated at room temperature for two hours before imaging. Rhodamine filter was used for imaging and the fluorescence signals were recorded without saturation. For the purpose of neuron identification by dye filling, both pre-dauer and post-dauer animals were mounted. Dauer animals were not used for this purpose because some amphid neurons are altered in shape and position in the dauer stage.

Single Molecule Fluorescent in situ Hybridization (smFISH)

smFISH was performed as described previously [11]. Pre-dauer larvae grown at 25℃ were used in the experiments. *hsp-4* probes were coupled to Cy5 and *daf-28* probes were coupled to Alexa Fluor 594. The oligonucleotide probes were designed based on the coding sequences of *hsp-4* and *daf-28,* using Stellaris FISH probe designer. To ensure specificity, *hsp-4* probes were designed to target the deleted region of the *hsp-4(gk514)* allele, and the probes yielded detectable signals in wild-type animals, but not in the *hsp-*

4(gk514) mutant (data not shown). The same strategy could not be adopted for *daf-28* probe design because the deleted region of the only deletion allele of *daf-28* available (*tm2308*) is too small. The designed *daf-28* probes allowed us to detect *daf-28* mRNA in the ASI and ASJ neurons, corroborating observations from previous studies using GFP transcriptional and translational reporters. Note, however, that *daf-28* mRNA expression levels are lower in ASJ (Figures S1A and S1B). Hybridized larvae were imaged using a Nikon Ti-E inverted fluorescence microscope equipped with a 100x oil-immersion objective and a Photometrics Pixis 1024 CCD camera using MetaMorph software and appropriate optical filters for Cy5, Alexa Fluor 594 and GFP. At least 10-20 animals per genotype were examined, and the observed *daf-28* and *hsp-4* mRNA expression patterns were derived from at least three independent experiments. ImageJ was used to perform maximum Z projection of stacked images. For image presentation, only linear adjustment (brightness and contrast) was applied equally to images from different genotypes to optimize signal-to-noise ratios for each channel.

Figure legends

Figure 1. The daf-28 (sa191) Mutation Causes ER Stress in the ASI Neuron Pair

(A) Fractions of the indicated *daf-28* mutants that enter the dauer stage at 25 °C. The *tm2308* mutation harbors a 158-base pair deletion that results in premature truncation of the DAF-28 peptide, while the *gk411702* mutation carries an early nonsense substitution mutation. Plotted is mean ± SD. The number of trials and animals scored is documented in Table S2.

(B) Fluorescence microscopy of the animals with indicated genotypes carrying the *hsp-4p::GFP* (*zcIs4*) transgene (GFP). Nomarski images (DIC) are provided for orientation. The amphid chemosensory neurons are identified by dye filling (DiI). The images shown are representative of three or more independent experiments.

(C) Maximum Z projection of smFISH stacked images of pre-dauer larvae with indicated genotypes. The ASI neurons are identified by GFP expression driven by the cell-specific *daf-7* promoter [18, 19].

See also Figures S1 and S2.

Figure 2. Activation of PERK/PEK-1 in the ASI Neuron Pair of the daf-28(sa191) Mutant Promotes the Dauer Developmental Decision

(A) Fractions of the *daf-28(sa191)* mutants with indicated *ire-1*, *xbp-1*, and *atf-6* deficiencies that enter the dauer stage at 25 °C.

(B) Fractions of the *daf-28(sa191)* mutants with indicated *pek-1* deficiencies that form dauer at 25 °C.

(C) Fractions of the indicated mutants in the *pek-1*-deficient background that form dauer at 25°C.

(D) Fractions of the animals with indicated genotypes that enter the dauer stage at 25 °C. The *daf-28(sa191);pek-1(ok275)* strain in this figure represents non-transgenic controls from all three lines. Results from the cell-specific *gpa-4* promoter are shown in this figure (referred to as P_{ASI}), and are confirmed with another ASI-specific promoter, *str-3p* (Figure S3B). The ASI neurons appeared intact in the animals where the cell-specific *pek-1* transgene was expressed (Figure S2C).

Plotted is mean ± SD. The number of trials and animals scored is documented in Table S2. See also Figure S3.

Figure 3. Phosphorylation of Ser49 of eIF2α by PEK-1 in the ASI Neuron Pair Promotes Entry into Dauer Diapause

(A) Fractions of the animals with indicated genotypes that enter the dauer stage at 25 °C. The *daf-28(sa191)* strain in this figure represents non-transgenic controls from all three lines.

(B) Fractions of the animals with indicated genotypes that enter the dauer stage at 25 °C. The *daf-28(sa191);pek-1(ok275)* strain in this figure represents non-transgenic controls from all three lines.

* $P < 0.01$ was determined by paired, two-tailed Student's *t*-test (non-transgenic fraction vs. transgenic fraction for each line). Results from the ASI-specific *gpa-4* promoter are shown in this figure (referred to as P_{ASI}).

Plotted is mean ± SD. The number of trials and animals scored is documented in Table S2. See also Figure S3.

(C) Schematic for the mechanism by which ER stress in the ASI neuron pair promotes entry into dauer diapause.

Supplemental figure legends are included with the figures.

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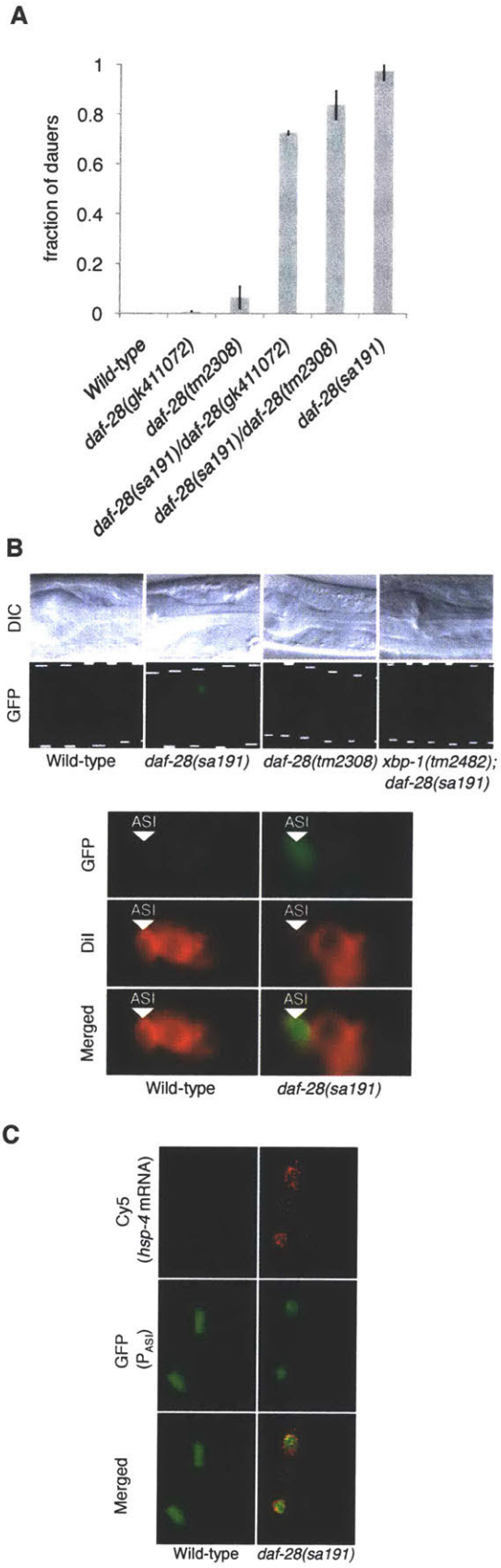
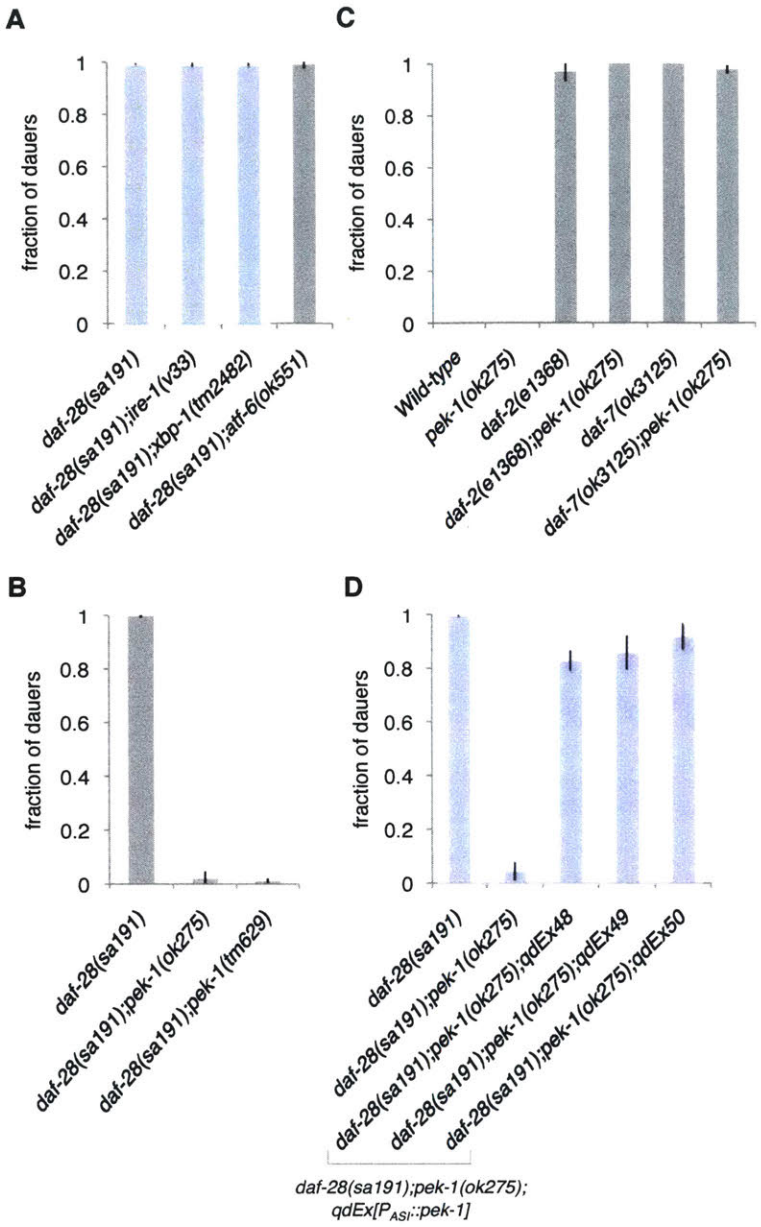

A
fraction of dauers
1
0.8
0.6
0.4
0.2
0
Wild-type
daf-28(gk411072)
daf-28(tm2308)
daf-28(sa191)/daf-28(gk411072)
daf-28(sa191)/daf-28(tm2308)
daf-28(sa191)
B
DIC
GFP
Wild-type
daf-28(sa191)
daf-28(tm2308)
xbp-1(tm2482); daf-28(sa191)
ASI
GFP
DiI
Merged
Wild-type
daf-28(sa191)
C
Cy5 (hsp-4 mRNA)
GFP (P ASI)
Merged
Wild-type
daf-28(sa191)

Figure 1


A
C
B
D
fraction of dauers
1
0.8
0.6
0.4
0.2
0
daf-28(sa191)
daf-28(sa191);ire-1(v33)
daf-28(sa191);xbp-1(tm2482)
daf-28(sa191);atf-6(ok551)
Wild-type
pek-1(ok275)
daf-2(e1368)
daf-2(e1368);pek-1(ok275)
daf-7(ok3125)
daf-7(ok3125);pek-1(ok275)
daf-28(sa191);pek-1(ok275)
daf-28(sa191);pek-1(tm629)
daf-28(sa191);pek-1(ok275);qdEx48
daf-28(sa191);pek-1(ok275);qdEx49
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daf-28(sa191);pek-1(ok275);
qdEx[P_ASI::pek-1]

Figure 2

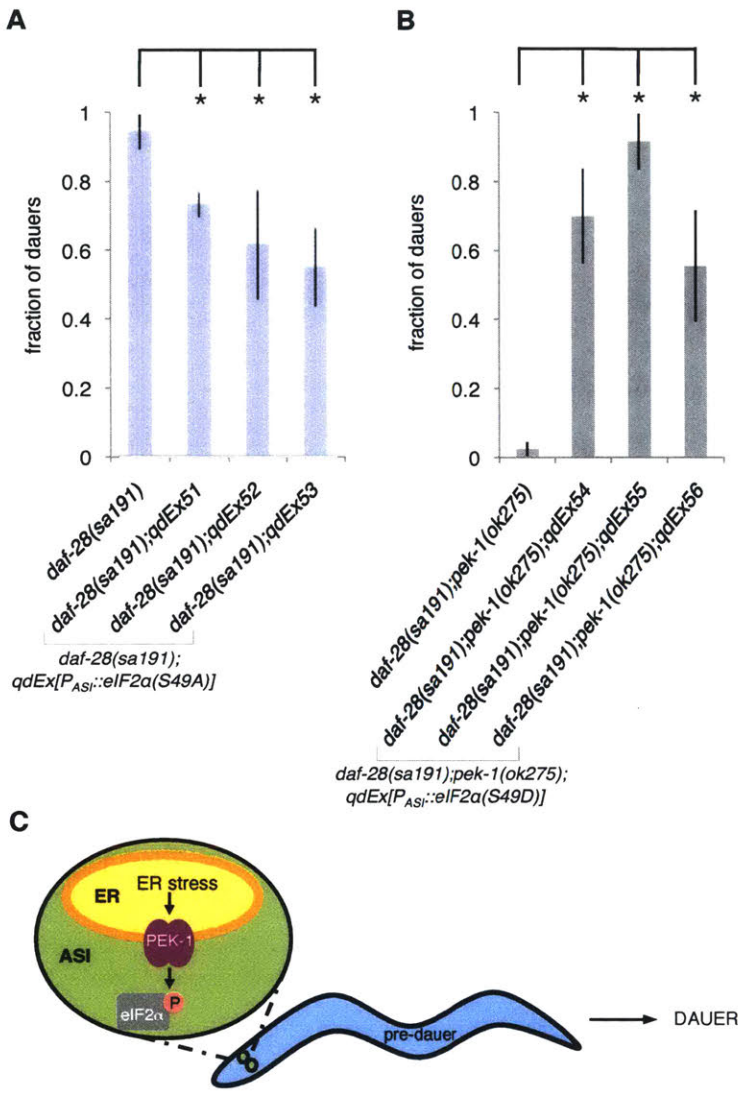
A
B
fraction of dauers
1
0.8
0.6
0.4
0.2
0
*
*
*
daf-28(sa191)
daf-28(sa191);qdEx51
daf-28(sa191);qdEx52
daf-28(sa191);qdEx53
daf-28(sa191);
qdEx[P_ASI::eIF2α(S49A)]
daf-28(sa191);pek-1(ok275)
daf-28(sa191);pek-1(ok275);qdEx54
daf-28(sa191);pek-1(ok275);qdEx55
daf-28(sa191);pek-1(ok275);qdEx56
daf-28(sa191);pek-1(ok275);
qdEx[P_ASI::eIF2α(S49D)]
C
ER
ER stress
PEK-1
ASI
eIF2α
P
pre-dauer
DAUER

Figure 3

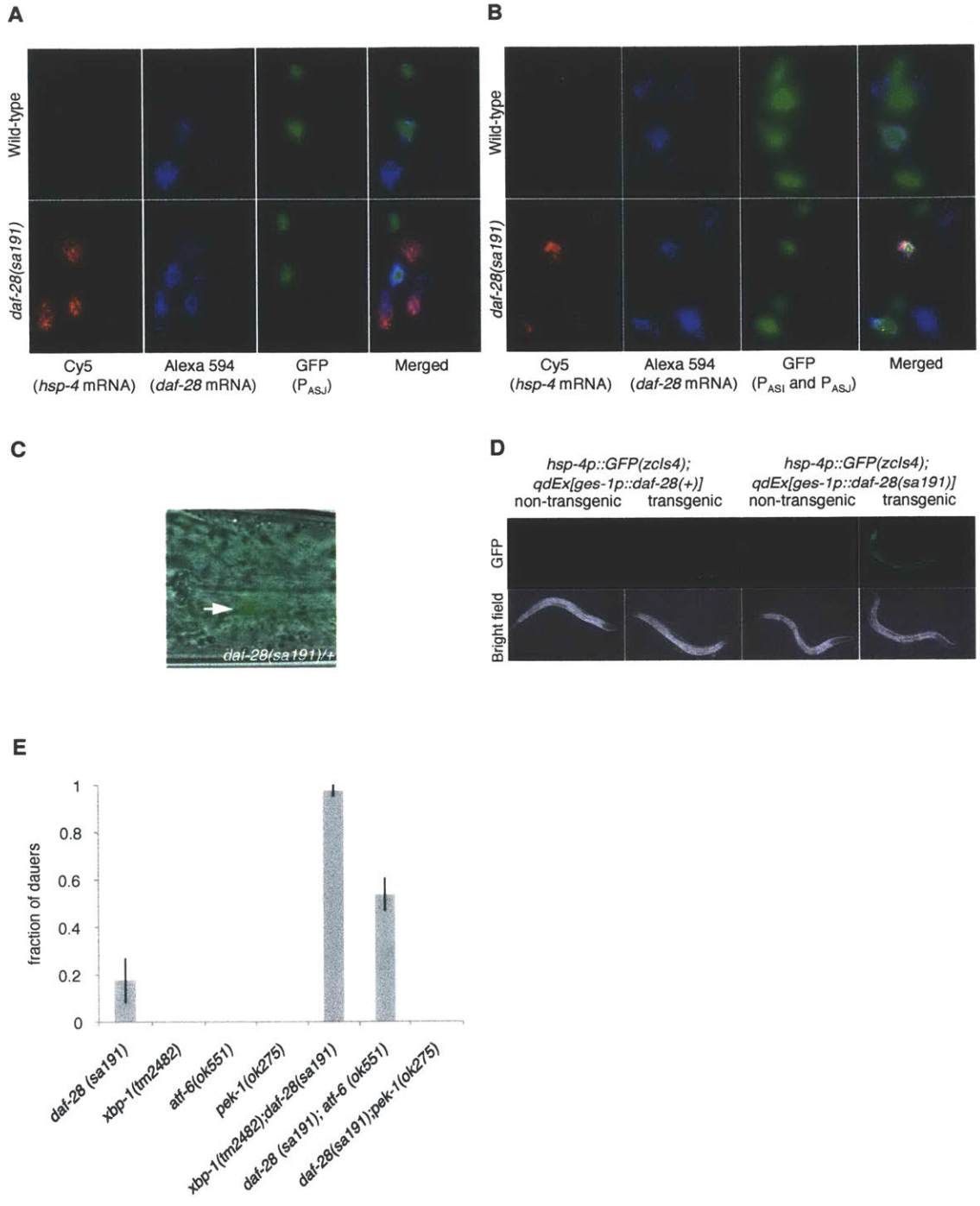
A
Wild-type
daf-28(sa191)
Cy5
(hsp-4 mRNA)
Alexa 594
(daf-28 mRNA)
GFP
(PASJ)
Merged
B
Wild-type
daf-28(sa191)
Cy5
(hsp-4 mRNA)
Alexa 594
(daf-28 mRNA)
GFP
(PASI and PASJ)
Merged
C
daf-28(sa191)/+
D
hsp-4p::GFP(zcIs4);
qdEx[ges-1p::daf-28(+)]
non-transgenic
transgenic
hsp-4p::GFP(zcIs4);
qdEx[ges-1p::daf-28(sa191)]
non-transgenic
transgenic
GFP
Bright field
E
fraction of dauers
1
0.8
0.6
0.4
0.2
0
daf-28 (sa191)
xbp-1(tm2482)
atf-6(ok551)
pek-1(ok275)
xbp-1(tm2482);daf-28(sa191)
daf-28 (sa191); atf-6 (ok551)
daf-28(sa191);pek-1(ok275)

Figure S1. The *daf-28 (sa191)* Mutation Causes ER Stress, Related to Figure 1

(A) Maximum Z projection of smFISH stacked images of pre-dauer larvae with indicated genotypes. The ASJ neurons are identified by expression of the translational fusion TRX-1::GFP [S1].

(B) Maximum Z projection of smFISH stacked images of pre-dauer larvae with indicated genotypes. The ASI and ASJ neurons are identified by GFP expression driven by the *daf-28* promoter [S2].

(C) Shown is fluorescence microscopy of the *daf-28(sa191)*/+ heterozygote that carries the *hsp-4p::GFP* transgene (green). The filled arrow indicates ASI.

(D) Shown is fluorescence microscopy of the animals with indicated genotypes that carry the *hsp-4p::GFP* transgene. *ges-1,* an intestine-specific promoter [S3], was used to drive ectopic expression of *daf-28*, both wild-type and *sa191* versions. *ofm-1p::GFP*, expressed in the coelomocytes, was used as a visible co-injection marker for transgenic animals. All images were recorded using the same exposure time.

Images shown represent five independent transgenic lines for *qdEx[ges-1p::daf-28(sa191)]*, all of which exhibit robust induction of *hsp-4p::GFP* expression in the intestine.

For *qdEx[ges-1p::daf-28(+)]*, images shown are representative of thirteen out of fourteen independent transgenic lines. One line carrying *qdEx[ges-1p::daf-28(+)]* exhibits modest and variable induction of *hsp-4p::GFP* expression in the intestine.

(E) Fractions of the *daf-28(sa191)* mutants with indicated *xbp-1, atf-6* and *pek-1* deficiencies that enter the dauer stage at 20 °C. Similar results were obtained using another loss-of-function allele of *xbp-1, tm2457*. Plotted is mean ± SD. The number of trials and animals scored is documented in Table S3.

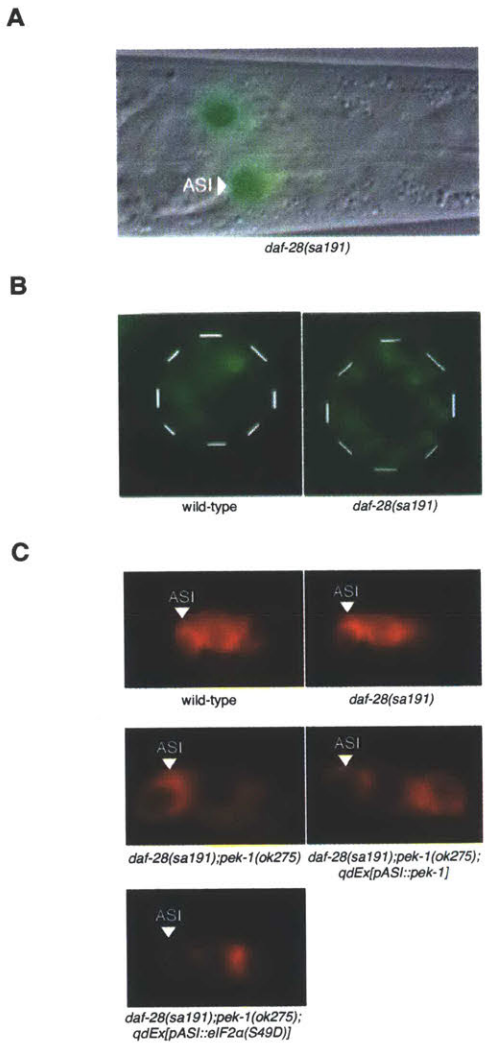

Figure S2. The *daf-28(sa191)* Mutation and Neuronal UPR Activation Does Not Compromise Cellular Survival and Function, Related to Figure 1

(A) Representative fluorescence microscopy of the anterior section of the *daf-28(sa191)* mutant carrying the *daf-28p::GFP* transgene. Note apparent expression of the transcriptional reporter (green).

(B) Representative fluorescence microscopy of the coelomocytes of the animals carrying the *daf-28p::DAF-28::GFP* transgene [S4] with indicated genetic backgrounds. Coelomocytes are specialized in taking up material from the pseudocoelomic fluid. Since *daf-28* is not expressed in the coelomocytes, the accumulation of DAF-28::GFP (note green puncta) could only be attributed to uptake from the pseudocoelom, where DAF-28::GFP, produced and secreted from the chemosensory neurons is deposited. The patterns depicted are representative of three independent experiments. Post-dauer animals were used in the experiments for consistency in coelomocyte identification.

(C) Fluorescence microscopy of the dye-filled amphid chemosensory neurons of the animals with indicated genotypes. The patterns depicted are representative of developmental stages both pre- and post-dauer entry at the restrictive temperature 25°C. For transgenic animals, images shown represent observations from three independent lines.

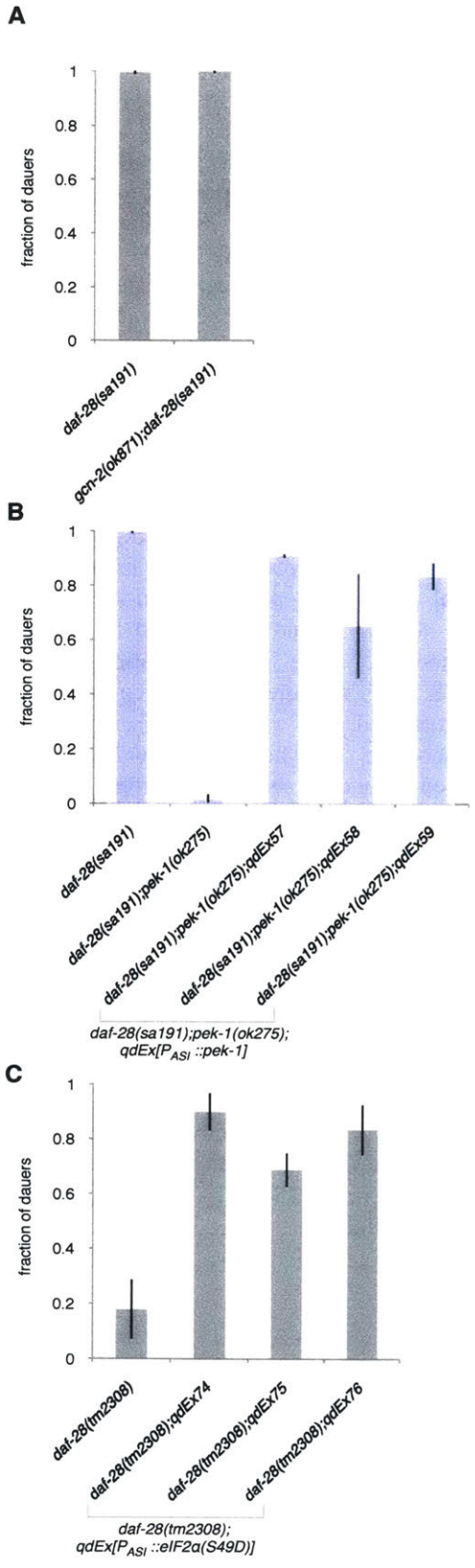

Figure S3. PEK-1 Phosphorylates eIF2α in the ASI Neuron Pair to Promote Entry into Dauer Diapause , Related to Figures 2 and 3

(A) Fractions of the animals with indicated genotypes that enter the dauer stage at 25 °C. Unlike PEK-1, GCN-2, a *C. elegans* ortholog of the eIF2α kinase GCN2 that functions independently of ER stress, is not required for the constitutive entry into dauer in the *sa191* animals. These observations rule out the possibility that general dysregulation of eIF2α phosphorylation promotes dauer entry, and pointing to an ER stress-specific role of PEK-1 in promoting the dauer developmental decision.

(B) Results from a second ASI-specific promoter, *str*-3p, are shown here (referred to as P_{ASI}). The *daf-28(sa191);pek-1(ok275)* strain in this figure represents non-transgenic animals from all three lines. Both *str-3p* and *gpa-4p* were characterized and validated in previous studies [S5-S7].

(C) Fractions of the animals with indicated genotypes that enter the dauer stage at 25 °C. The *daf-28(tm2308)* strain in this figure represents non-transgenic controls from all three lines. Results from the ASI-specific *gpa-4* promoter are shown in this figure (referred to as P_{ASI}).

Plotted is mean ± SD. The number of trials and animals scored is documented in Table S3.

Table S1. Data for Dauer Formation Assays, Related to Figure 1 and Discussion

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	97.8 ± 3.9	19	1017
daf-16(mgDf47);daf-3(mgDf90)	0 ± 0	10	690
daf-16(mgDf47);daf-28(sa191);daf-3(mgDf90)	38.4 ± 10.4*	8	371

Note: The *daf-16(mgDf47);daf-28(sa191);daf-3(mgDf90)* animals only formed partial dauers (*), the features of which were described in [S8]. A previous study showed that *daf-16;daf-28(sa191)* mutants only formed partial dauers, and the fraction of partial dauers was significantly lower than the fraction of *bona fide* dauers formed by the *daf-28(sa191)* mutant [S9].

Table S2. Data for Dauer Formation Assays, Related to Figures 1, 2 and 3

Figure 1A

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
N2	0 ± 0	7	221
daf-28(gk411072)	0.6 ± 0.5	6	657
daf-28(tm2308)	6.3 ± 4.8	19	887
daf-28(gk411072)/daf-28(tm2308)	0.4 ± 1.3	10	183
daf-28 (sa191)/daf-28 (gk411072)	72.5 ± 1.0	5	77
daf-28 (sa191)/daf-28 (tm2308)	83.7 ± 6.0	9	399
daf-28(sa191)	97.5 ± 4.0	16	857

Note: We observed minimal or no Daf-c phenotype in animals carrying two putative null alleles of *daf-28*, the *daf-28(tm2308)* [S10] and *daf-28(gk411072)* alleles that were not available at the time of prior studies, indicating that the robust Daf-c phenotype of the *sa191* mutant cannot be attributed to loss of function of the insulin-encoding gene *daf-28*. In addition, the trans-heterozygote between the *sa191* mutation and each of the *daf-28* null alleles exhibited constitutive entry into dauer similar to the *sa191* homozygote, corroborating the reported gain-of-function nature of the *sa191* mutation.

Figure 2A

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.7 ± 0.5	8	665
daf-28(sa191);ire-1(v33)	99.6 ± 1.1	7	264
daf-28(sa191);xbp-1(tm2482)	99.6 ± 1.0	7	357
daf-28(sa191);atf-6(ok551)	99.1 ± 1.4	8	595

Figure 2B

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.7 ± 0.5	8	665
daf-28(sa191);pek-1(ok275)	1.9 ± 2.8	9	891
daf-28(sa191);pek-1(tm629)	0.9 ± 1.2	6	456

Figure 2C

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
N2	0 ± 0	8	264
pek-1(ok275)	0 ± 0	7	537
daf-2(e1368)	96.8 ± 3.5	7	325
daf-2(e1368);pek-1(ok275)	100 ± 0	4	237
daf-7(ok3125)	100 ± 0	2	97
daf-7(ok3125);pek-1(ok275)	97.8 ± 1.5	6	411

Figure 2D

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.7 ± 0.5	8	665
daf-28(sa191);pek-1(ok275)	4.4 ± 3.45	12	542
daf-28(sa191);pek-1(ok275);qdEx48	82.8 ± 3.9	4	103
daf-28(sa191);pek-1(ok275);qdEx49	85.9 ± 6.4	4	194
daf-28(sa191);pek-1(ok275);qdEx50	91.9 ± 5.0	4	278

Figure 3A

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	94.1 ± 5.1	13	399
daf-28(sa191);qdEx51	73.1 ± 3.6	4	141
daf-28(sa191);qdEx52	67.9 ± 7.6	4	169
daf-28(sa191);qdEx53	54.8 ± 11.4	4	119

Figure 3B

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191);pek-1(ok275)	2.4 ± 2.0	14	1037
daf-28(sa191);pek-1(ok275);qdEx54	69.9 ± 1.4	5	254
daf-28(sa191);pek-1(ok275);qdEx55	91.5 ± 8.0	5	117

Table S3. Data for Dauer Formation Assays, Related to Figures 3, S1, S2 and S3 and Discussion

Related to Figure 3

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	98.3 ± 2.4	10	536
daf-28(sa191);qdEx60	98.1 ± 1.3	4	139
daf-28(sa191);qdEx61	98.4 ± 1.4	3	135
daf-28(sa191);qdEx62	98.3 ± 2.9	3	54
daf-28(sa191);pek-1(ok275)	0.1 ± 0.4	12	612
daf-28(sa191);pek-1(ok275);qdEx71	0.4 ± 0.8	4	196
daf-28(sa191);pek-1(ok275);qdEx72	0 ± 0	4	118
daf-28(sa191);pek-1(ok275);qdEx73	0 ± 0	4	156

Note: *qdEx60*, *qdEx61*, *qdEx62*, *qdEx71*, *qdEx72* and *qdEx73* represent *qdEx[gpa-4p::eIF2α(+)::unc-54 3'UTR]*.

Figure S1E

Genotype	Mean percentage of dauers ± standard deviation (percentage) at 20 °C	Number of trials	Number of total (pooled) animals
daf-28(sa191)	17.6 ± 9.3	7	511
xbp-1(tm2482)	0 ± 0	4	228
atf-6(ok551)	0 ± 0	4	280
pek-1(ok275)	0 ± 0	4	274
xbp-1(tm2482);daf-28(sa191)	97.6 ± 2.4	5	228
daf-28(sa191);atf-6(ok551)	53.7 ± 6.9	6	280
daf-28(sa191);pek-1(ok275)	0 ± 0	6	304

Figure S3A

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.6 ± 0.6	8	659
gcn-2(ok871);daf-28(sa191)	99.8 ± 0.5	6	427

Figure S3B

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.7 ± 0.5	8	665
daf-28(sa191);pek-1(ok275)	1.4 ± 2.0	12	591
daf-28(sa191);pek-1(ok275);qdEx57	90.8 ± 0.7	4	121
daf-28(sa191);pek-1(ok275);qdEx58	65.2 ± 19.2	4	102
daf-28(sa191);pek-1(ok275);qdEx59	83.4 ± 4.8	4	246

Figure S3C

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(tm2308)	17.9 ± 10.7	14	412
daf-28(tm2308);qdEx74	89.9 ± 6.7	5	154
daf-28(tm2308);qdEx75	68.7 ± 6.0	4	146
daf-28(tm2308);qdEx76	83.3 ± 9.1	5	122

Related to Figure S2 and Discussion

Genotype	Mean percentage of dauers ± standard deviation (percentage)	Number of trials	Number of total (pooled) animals
daf-28(sa191)	99.6 ± 0.6	6	493
daf-28(sa191);daf-3(mgDf90)	99.8 ± 0.5	6	564

Note: DAF-3/SMAD is required for chemosensory neuron ablation to mediate dauer formation [S11]. The result is consistent with a previous genetic study [S9] using a different allele of *daf-3*, namely *e1376*.

Chapter III

Neuronal eIF2α Phosphorylation Mediates Developmental Plasticity and Organismal Stress Responses

This chapter is adapted from an ongoing manuscript to be submitted for publication. The section describing the suppressor screen and the genes associated has been expanded here.

Abstract

Phosphorylation of the alpha subunit of eIF2 (eIF2α) is an evolutionarily conserved mechanism regulating eukaryotic translational initiation in response to endogenous and environmental cues at the cellular level. Recent studies have also highlighted the cell-non-autonomous roles of eIF2α phosphorylation in the mammalian nervous system in mediating complex processes such as feeding behavior and memory formation (Hao et al., 2005 and Costa-Mattioli et al., 2007). In *Caenorhabditis elegans*, phosphorylation of serine 49 on eIF2α in two chemosensory neurons by the eIF2α kinase PEK-1/PERK triggers a systemic response, specifically by promoting entry into a stress-resistant, long-lived developmental stage termed dauer (Kulalert and Kim, 2013). Here, we report the identification of molecular determinants regulating sensitivity to neuronal eIF2α phosphorylation. We isolated mutations in the gene encoding the alpha subunit of eIF2B that suppress the organismal consequences of neuronal eIF2α phosphorylation, supporting a conserved role for eIF2Bα in providing a binding site for phosphorylated eIF2α to inhibit the exchange factor eIF2B catalytic activity that is required for

translation initiation. We also recovered a novel mutation that confers systemic resistance to eIF2α phosphorylation in the gene encoding the gamma subunit of eIF2. The eIF2γ mutation not only suppresses sensitivity to the effect of phosphorylated eIF2α, but also renders a subunit of eIF2B that is essential in the wild type background dispensable. Having identified the molecular and cellular determinants of sensitivity to eIF2α phosphorylation, we proceed to show that constitutive expression of eIF2α carrying a phosphomimetic S49D mutation in the ASI neurons alters the organismal sensitivity to the physiological dauer-inducing cues, specifically by enhancing dauer entry through bypassing the requirement for a pro-dauer food source in the presence of the dauer pheromone. Furthermore, ectopic phosphorylation of eIF2α in the ASI neuron pair confers dramatic cell-nonautonomous effects on growth, metabolism and reproduction in adult animals, phenocopying organismal responses to starvation. Our study establishes that conserved molecular determinants regulate sensitivity to the organismal effects of neuronal eIF2α phosphorylation in *C. elegans*, and suggests that modulation of eIF2α phosphorylation can have dramatic cell-nonautonomous effects on developmental plasticity and organismal physiology.

Introduction

eIF2α phosphorylation: an evolutionarily conserved translational control mechanism

Phosphorylation of the alpha subunit of eIF2 (eIF2α) is an evolutionarily conserved mechanism of translation control in eukaryotic cells that is pivotal for regulation of gene expression during stress (reviewed in Sonenberg and Hinnebusch, 2009). In *Saccharomyces cerevisiae*, eIF2α phosphorylation mediated by the GCN2 kinase governs

cellular adaptations in response to nutrient deficiency, specifically by attenuating global protein synthesis and preferentially upregulating translation of transcripts that are associated with stress alleviation, notably the transcription factor GCN4 (Hinnebusch, 2005). In mammals, four eIF2α kinases have been identified and are activated by endogenous and environmental cues that include amino acid starvation (GCN2), endoplasmic reticulum (ER) protein-folding imbalance (PERK), presence of foreign double-stranded RNA (PKR) and heme deprivation (HRI), hence constituting a homeostatic mechanism termed the Integrated Stress Response (ISR) (reviewed in Sonenberg and Hinnebusch, 2009).

While the biochemical consequences of eIF2α phosphorylation have been thoroughly delineated at the cellular level (reviewed in Wek et al., 2006, reviewed in Sonenberg and Hinnebusch, 2009), recent studies have also demonstrated cell nonautonomous and tissue-specific roles of phosphorylation of eIF2α in animal physiology and human diseases. For instance, eIF2α phosphorylation and the eIF2α kinase GCN2 have been shown to regulate intestinal homeostasis and suppress gut inflammation (Cao et al., 2014 and Ravindran et al., 2016). Recent findings have also shed light on physiological roles of eIF2α phosphorylation in the mammalian central nervous system, regulating complex processes such as foraging behavior and memory formation. Specifically, essential amino acid deprivation induces phosphorylation of eIF2α via GCN2 in the mammalian anterior piriform cortex to promote aversion to an amino acid-deficient diet (Hao et al., 2005 and Maurin et al., 2005). Neuronal eIF2α phosphorylation also governs synaptic plasticity and learning by modulating expression of proteins involved in both long-term potentiation and depression at hippocampal

synapses (Costa-Mattioli et al., 2007 and Di Prisco et al., 2014). Furthermore, human genetic analyses have revealed that mutations in genes encoding translation initiation components regulating eIF2 activity such as subunits of the exchange factor eIF2B and the gamma subunit of eIF2 are associated with defects in myelination in the brain and mental disability, respectively (reviewed in Bugiani et al., 2010, Borck et al., 2012). Collectively, these findings indicate that, in addition to maintaining cellular homeostasis and viability, eIF2α phosphorylation state in the nervous system can have a major influence on animal physiology and pathology.

The daf-28(sa191) Daf-c suppressor screen

In Chapter II, I characterized the mechanism by which the *daf-28(sa191)* mutation confers constitutive dauer entry. The R37C substitution in the DAF-28 insulin peptide causes endoplasmic reticulum stress specifically in the ASI chemosensory neurons, activating the Unfolded Protein Response regulator PEK-1/PERK, which phosphorylates a conserved regulatory Ser49 in eIF2α in the ASI chemosensory neuron pair to promote entry into dauer diapause (Kulalert and Kim, 2013). To further identify the mechanism underlying the organismal response to this neuron-specific eIF2α phosphorylation, we sought to isolate mutations that could suppress the constitutive dauer entry phenotype of the *daf-28(sa191)* mutant.

By adopting the dauer experimental system to genetically dissect the mechanisms underlying organismal stress adaptations that are mediated by neuronal eIF2α phosphorylation, we have uncovered systemic roles of conserved translational regulatory factors and revealed dramatic physiological consequences that can arise from the aberrant

control of translation in just a pair of *C. elegans* sensory neurons.

Results

Summary of the alleles recovered from the daf-28(sa191) Daf-c suppressor screen

We mutagenized approximately 40,000 genomes of the *daf-28(sa191)* animals and isolated suppressor mutants that failed to enter dauer constitutively under the same conditions that induce the starting strain to enter diapause (Figure S1). We then employed complementation testing and whole genome sequencing to identify candidate genes. We categorized the isolated mutants into three distinct classes:

Class I: mutations that modulate the biosynthesis of the misfolded DAF-28 and suppress subsequent activation of the UPR/PEK-1

Class II: mutations that suppress the consequences of phosphorylation of eIF2α

Class III: mutations that suppress neuroendocrine responses required for the dauer decision

Class I and Class II genes are shown in Figures 1 and S1. *dpy-3, dpy-8* and *rol-3*, which belong to Class I, will be described and discussed in Chapter IV. Class III genes include alleles of *daf-2, pkc-1,* and two other genes, and will be described in Chapter IV.

Class I gene: *daf-28*

Consistent with the model indicating that production of a toxic DAF-28 peptide triggers entry into dauer diapause, we isolated second-site, revertant mutations in the gene encoding the toxic DAF-28 insulin. We identified a presumptive null allele of *daf-28,* which carries a missense mutation in the start codon (M1X R37C), preventing

translation initiation of the peptide. The absence of the toxic DAF-28 peptide likely abrogates ER protein folding imbalance that triggers neuronal eIF2α phosphorylation and dauer formation through PEK-1. Consistent with such hypothesis, we note that this allele of *daf-28* behaves similarly to the null *daf-28(tm2308)* deletion allele (Figure S2 and Kulalert and Kim, 2013). We also isolated another allele of *daf-28,* which results in a premature truncation of the peptide (R37C Q81X). Since we observed similarities in genetic behaviors between this allele and wild-type *daf-28* (Figure S2 and Kulalert and Kim, 2013), we speculate that the 16-amino-acid truncation, while having no effect on DAF-28 function, results in an even number of cysteine residues that might restore proper cysteine pairing, thus abolishing neuronal ER toxicity that promotes the dauer decision.

Class I gene: *ERp44/c30h7.2*

In addition, we isolated three presumptive null alleles of a previously uncharacterized gene *c30h7.2* (Figures 1 and S1). The suppression of the dauer entry phenotype was also confirmed by a previously generated null allele of *c30h7.2, gk411949* (fraction of dauers = 0.02 ± 0.02; N = 228). The gene encodes an ortholog of mammalian ERp44, which is a member of the protein-disulfide isomerase family (Anelli et al., 2002 and Anelli et al., 2003). ERp44 is involved in quality control of several ER client proteins (Higo et al., 2005, Freyaldenhoven et al., 2012, Hisatsune et al., 2015, Yang et al., 2016). Importantly, ERp44 has been shown to directly interact with proinsulin in mouse insulinoma cells and its expression is induced in the dysfunctional insulin-producing beta cells of the diabetes rat model (Pottekat et al., 2013 and Yang et al., 2016). We hypothesized that ERp44 may participate in formation or retention of the toxic DAF-28

mutant product that activates the Unfolded Protein Response (UPR) to promote dauer entry. Consistent with this hypothesis, we observed reduction of the neuronal induction of the IRE1-XBP1 arm of the UPR in the absence of ERp44, suggesting that the toxicity caused by the mutant neuronal insulin is in part mediated by the isomerase (Figure 1C). It is also plausible that the absence of ERp44 may result in compensatory upregulation of other resident chaperones that generally improve ER homeostasis and mitigate the toxicity triggered by the mutant insulin. While we recovered three presumptive null alleles of *c30h7.2*, we did not identify any suppressor mutations in the other two *C. elegans* paralogs of *c30h7.2*, *c06a6.5* and *f42g8.7*, suggesting specificity of this particular isoform of ERp44 in mediating insulin folding and/or neuronal ER homeostasis.

Class I gene: *pek-1*

We also isolated eighteen new alleles of *pek-1,* which encodes the *C. elegans* ortholog of mammalian eIF2α kinase PERK that is activated by the toxic peptide-mediated disruption of protein folding homeostasis in the ER, consistent with our previous study (Figures 1, S1 and Kulalert and Kim, 2013). In addition to nonsense substitutions that lead to premature truncation of PEK-1, the majority of the recovered *pek-1* alleles harbor alterations in the conserved residues in the cytoplasmic kinase domain of PEK-1 (Figure S3).

Class II gene: *eIF2α/eif-2.1*

Importantly, we recovered an allele of the essential gene encoding the *C. elegans* ortholog of eIF2α (*y37e3.10*) that results in an S49F substitution at the conserved Ser49

target of eIF2α kinases (Figures 1A and 1B). The suppression of the dauer entry phenotype by this *eIF2α/eif-2.1* allele further corroborates our previous observation that neuron-specific expression of the unphosphorylatable version of *eIF2α* could partially inhibit dauer entry in the *daf-28(sa191)* background (Kulalert and Kim, 2013).

The remaining class II genes (*eif-2B.1* and *eif-2.3*) will be discussed in the remaining sections of this chapter.

eIF2Bα mediates organismal sensitivity to neuronal eIF2α phosphorylation in C. elegans

Translation initiation requires guanine nucleotide exchange on eIF2 mediated by the multimeric eIF2B (reviewed in Sonenberg and Hinnebusch, 2009). We isolated eleven distinct suppressor mutations in the gene encoding the *C. elegans* ortholog of the alpha subunit of eIF2B (*eif-2B.1*), *zk1098.4* (Figures 1B and 2). Two presumptive null alleles of *eif-2B.1* also suppressed the constitutive dauer entry phenotype (Figure 2A). Most of the *eif-2B.1* alleles isolated conferred the suppression in a dominant fashion, similar to the *eif-2B.1* deletion allele (Figure S4). Molecular genetic studies in yeast have established that eIF2Bα is a non-essential, regulatory subunit that provides a binding site for phosphorylated eIF2α (eIF2(αP)) to inhibit the eIF2B Guanine Exchange Factor (GEF) activity required for translation initiation (Yang and Hinnebusch, 1996).

We sought to correlate the suppression of the constitutive dauer entry phenotype with an independent measure of resistance to phosphorylation eIF2α. Induction of the IRE-1-XBP-1 arm of the UPR has been observed in the spermatheca (Calfon et al., 2002), possibly due to the demanding role of the ER in regulating calcium-mediated ovulation (Bui and Sternberg, 2002). Using a transgenic reporter harboring the ATF-5

upstream regulatory region fused to GFP, we observed robust fluorescence in the spermatheca, consistent with activation of the eIF2α kinase-ATF-5 pathway. We noted that the increased fluorescent reporter expression was dependent on the phosphorylation of Ser49, as spermatheca fluorescence was diminished in the *eif-2.1(qd338)* mutant background where Ser49 is altered (Figure 2B). Consistent with the role of eIF2Bα in mediating the cellular effects of eIF2α phosphorylation, eIF2(αP)-dependent ATF-5 induction in adult spermatheca observed in the wild-type animals was substantially diminished in the *C. elegans eIF2Bα* mutant background (Figure 2B).

Our data suggest a conserved molecular target and mechanism in response to eIF2α phosphorylation at both cellular (Figure 2B) and organismal (Figure 1B) levels in *C. elegans*. We observed that the beta and delta subunits of eIF2B, unlike the alpha subunit, are essential for *C. elegans* development (Figure 3B).

A mutation conferring altered function in eIF2γ confers resistance to neuron-specific eIF2α phosphorylation in C. elegans

The translation factor eIF2 is a G-protein complex composed of three distinct subunits, alpha, beta and gamma (reviewed in Sonenberg and Hinnebusch, 2009). We identified a suppressor mutation in the *C. elegans* ortholog of the gamma subunit of eIF2 (*eif-2.3*), *y39g10ar.8* (Figures 1A and 1B), which conferred dauer suppression in a dominant manner, suggesting alteration of function of this essential factor. The mutation alters the conserved serine residue in domain III of eIF2γ (Roll-Mecak et al, 2003), resulting in an S443L substitution. We observed that eIF2(αP)-dependent ATF-5 reporter induction in adult spermatheca was significantly reduced in the *eif-2.3* mutant

background, further indicating that the mutation contributes to insensitivity to eIF2α phosphorylation (Figure 3C).

The isolation of an unusual allele of *eif-2.3* that can confer insensitivity to the effects of eIF2(αP) was reminiscent of a previously characterized mutation in *GCD11*, the yeast ortholog of *eIF2γ*, which obviates the need for the GEF eIF2B in protein synthesis (Erickson and Hannig, 1996 and Erickson et al., 2001). We examined if the *eif-2.3(qd336)* mutation could also enable eIF2B-independent translation initiation by performing RNAi knockdown of individual eIF2B subunits to assess whether the *eIF2γ* mutant can bypass the requirement for eIF2B during larval development. RNAi knockdown of the essential regulatory subunits, eIF2Bβ and eIF2Bδ, and catalytic subunits, eIF2Bγ and eIF2Bε, inhibited larval development in the wild-type background (Figure 3B). Strikingly, we observed that the *eif-2.3(qd336)* mutant developed normally and reached reproductive maturation in the absence of eIF2Bγ, while still failing to survive when expression of the other essential eIF2B subunits had been impaired by RNAi (Figures 3A and 3B).

The ability of the *C. elegans eIF2γ* mutant to specifically bypass the requirement for eIF2Bγ indicates that the S443L change may result in biochemical alterations in eIF2γ that would otherwise require catalysis by eIF2Bγ, allowing proper translation initiation and developmental viability in the absence of the eIF2B subunit. The mutant EIF-2.3 still requires a GEF for efficient eIF2 recycling because eIF2Bε, the key catalytic subunit for the nucleotide exchange activity, is still necessary for larval viability in the *eif-2.3* mutant background (Figure 3B). We have demonstrated that a subset of eIF2B catalytic activities can be dispensable in the *eif-2.3(qd336)* mutant background (Figures 3A and 3B), likely

resulting in diminished dependence on eIF2B and thus liberating eIF2 recycling from the inhibitory effects of eIF2(αP)-mediated eIF2B sequestration. We note that the ability to circumvent the eIF2Bγ requirement is unique to the *eif-2.3* mutant, and does not occur in other eIF2(αP)-insensitive mutants (Figure S5). The S443L substitution in EIF-2.3 also confers insensitivity to eIF2α phosphorylation via a distinct mechanism from the aforementioned *S. cerevisiae GCD11(K250R)* mutation (Erickson and Hannig, 1996 and Erickson et al., 2001), as the *C. elegans eIF2γ* mutation did not result in general growth impairment or bypass of the essential functions of eIF2α and all essential eIF2B subunits (Figures 3B and S8, compared to Erickson et al., 2001).

Examination of the physiological roles of sensitivity to eIF2α phosphorylation

We then asked whether the organismal sensitivity to eIF2α phosphorylation is required for the dauer developmental decision in response to stimuli beyond misfolded neuropeptides and neuronal ER stress. The eIF2(αP)-insensitive mutants are not dauer-defective, as they were able to form dauers when the plates were starved out and crowded, similarly to wild-type control (Figure S6), indicating that the ability to respond to phosphorylation of eIF2α is dispensable for the developmental decision upon encountering a combination of stressors that include crowding and nutritional deprivation. Upon exposure to a combination of synthetic dauer-inducing ascarosides (collectively termed dauer pheromone), while food is abundant albeit of poor quality (heat-killed *E. coli*; see Methods), both wild-type and eIF2(αP)-insensitive mutants formed dauers efficiently (Figure S6), demonstrating that, under the standard assay condition, pheromone perception and downstream signal transduction mechanisms

operate independently of eIF2α phosphorylation responsiveness. Insensitivity to eIF2(αP) also had no effect on the dauer-constitutive insulin receptor mutant (Figure S6). Collectively, these observations suggest that eIF2α phosphorylation does not have a significant non-redundant role in dauer entry in response to the dauer pheromone and diminished insulin neuroendocrine signaling.

eIF2α phosphorylation is a homeostatic mechanism to integrate a broad array of cellular stressors including starvation and proteostasis imbalance in *C. elegans* (Richardson et al., 2011 and Rousakis et al., 2013). We therefore asked whether the inability to respond to phosphorylation of eIF2α contributes to other organismal phenotypes beyond the dauer developmental decision. Because *Pseudomonas aeruginosa* infection has been shown to activate the innate immune system that imposes secretory burdens to the ER (Richardson et al., 2010), we asked if proper translational regulation was required for larval development in the presence of the pathogen. We observed that both the wild-type and eIF2(αP)-insensitive animals developed normally upon exposure to *Pseudomonas aeruginosa* (Figure S7). Stressors associated with ER stress, such as the drug tunicamycin and heat, that may activate the eIF2α kinase PEK-1 did not elicit distinguishable responses between wild-type control and the eIF2(αP)-insensitive mutant (Figure S7). Loss-of-function mutations of *pek-1* have been shown to impair larval viability at 25°C in the genetic background where another UPR arm IRE-1-XBP-1 is disrupted (Shen et al., 2005 and Richardson et al., 2011). We observed that insensitivity to eIF2α phosphorylation downstream of PEK-1 did not result in the synthetic larval arrest phenotype with *xbp-1* mutation (N = 313), suggesting that the compensatory effects

of PEK-1 in maintaining proteostasis in the absence of XBP-1 may be largely eIF2(αP)-independent.

Overexpression of phosphomimetic S49D eIF2α in the ASI neuron pair alters the larval sensitivity to physiological stimuli that induce dauer formation

We then asked whether constitutive phosphorylation of eIF2α has any impact on the organismal physiology. Ectopic expression of phosphomimetic eIF2α in all tissues would result in global translational attenuation that impairs growth, similar to the effects of RNAi knockdown of essential eIF2 subunits (Figure 3B). We thus limit the scope of this study to constitutive ASI-specific eIF2α phosphorylation, which we have previously shown can promote dauer entry in some sensitized genetic backgrounds (Kulalert and Kim, 2013). We generated an integrant line carrying the *qdIs10* transgene expressing phosphomimetic *C. elegans eIF2α(S49D)* driven under the ASI-specific promoter *gpa-4p*. We observed that the animals carrying the *qdIs10* transgene, while not exhibiting the constitutive entry phenotype at the standard assay population density, were more prone to entering dauers when the plates became crowded without food deprivation (data not shown). Additionally, the *qdIs10* transgene was sufficient to enhance dauer formation in the insulin- and TGFβ-deficient genetic backgrounds, which partially sensitize the animals to form dauer larvae (Figure 4A). Overexpression of wild-type eIF2α, unlike the phosphomimetic variant, has been shown to have no effect on the dauer decision (Kulalert and Kim, 2013), indicating that the developmental effect elicited by the *qdIs10* transgene is phosphorylation-status-specific. Taken together, these observations suggest that, during larval development, ASI-specific eIF2α phosphorylation synergizes with

other previously characterized dauer-promoting inputs including crowding and aberrant neuroendocrine signaling levels to trigger entry into diapause.

The most robust non-genetic approach to induce dauer formation in the laboratory is treatment with assortments of ascarosides in the presence of heat-killed *E. coli* (Jeong et al., 2005, Kim et al., 2009, McGrath et al., 2011 and Park et al., 2012). The molecular understanding of the ability of heat-killed bacteria, but not live bacteria, to efficiently induce dauer entry in the presence of the dauer pheromone has been lacking. We noted that non-dauer animals grown on heat-killed *E. Coli* exhibited impaired growth and reproduction (data not shown), indicative of exposure to a poor nutritional source. Since the dauer decision is triggered by adverse conditions that include limited food availability, it is plausible that the nutritionally deficient nature of heat-killed bacteria contributes to robust dauer formation observed in the pheromone assay. Alternatively, or additionally, the microbe-derived chemosensory cues that the worm has evolved to associate with food sources of varied nutritional qualities may influence the dauer decision. Because inhibition of translation in *C. elegans* has been shown to be induced by nutrient deficiency and pathogen-derived toxin (Rousakis et al., 2013 and Dunbar et al., 2012), we hypothesized that translational attenuation via constitutive eIF2α phosphorylation in the ASI neurons that integrate both the dauer pheromone and the food signals may evoke perception of inferior, anti-growth food sources, mimicking the effects of heat-killed bacteria that potentiate dauer formation. Corroborating our hypothesis, the animals carrying the ASI-specific phosphomimetic eIF2α transgene formed dauers even in the presence of the dauer pheromone and live bacteria, the pro-growth food source that largely suppressed dauer entry in wild-type control (Figure 4B). The ability of the *qdIs10*

transgene to promote dauer formation in response to the dauer pheromone despite the presence of the pro-growth live *E. coli* is dependent on the organismal sensitivity to ectopic neuronal eIF2α phosphorylation mediated by eIF2Bα and eIF2γ, as the corresponding eIF2(αP)-insensitive mutations suppressed the *qdIs10* pro-dauer effects (Figure 4B). The ability of ectopic neuronal eIF2(αP) to mimic the pro-dauer effects despite the presence of the pro-growth food sources suggests that modulation of the eIF2α phosphorylation status in ASI can alter perception of the microbial and nutritional environment that affects developmental choices.

Overexpression of phosphomimetic S49D eIF2α in the ASI neuron pair mimics the effects of starvation

Because the dauer decision is a response to unfavorable conditions, we asked whether expression of phosphomimetic eIF2α that mimics the pro-dauer effects also adversely influences the physiology of non-dauer animals in other life stages. In adult hermaphrodite animals, neuronal expression of phosphomimetic eIF2α resulted in small body size (Figures 5A and 5B), consistent with diminished growth rate (Figure 5C). The animals expressing S49D eIF2α in the ASI neurons also exhibited clear appearances, consistent with diminished fat storage observed (Figure 5D). We also noted a significantly reduced brood size and an extension of the egg-laying period in the animals carrying the *qdIs10* transgene, in comparison to the wild-type control (Figures 5E and 5F). Unlike wild-type animals that stored 10-15 fertilized eggs *in utero*, the animals with ectopic neuronal eIF2α phosphorylation harbored a drastically reduced number of fertilized embryos (Figure 5G). The impairment in development and progeny production

was independent of rearing temperature. Notably, these reproductive and metabolic defects are reminiscent of those occurring in animals that were defective in feeding or subjected to unfavorable conditions that promote reallocation of resources and germ cell death, such as starvation (Avery, 1993 and Angelo and Van Gilst, 2009). Specifically, slow growth and extension of the reproductive period were observed in the *eat-2* mutant that is defective in food intake (Figure S8). Corroborating the cell-non-autonomous influence of neuronal eIF2α phosphorylation, we observed downregulation of *atgl-1*, which encodes a lipase that functions in the intestine (Figure 5H). Such alteration in gene expression in distal tissues may suggest an adaptive role of the organismal consequences of neuron-specific eIF2(αP), for instance in preventing exhaustion of the energy reserve.

Overexpression of wild-type and unphosphorylatable versions of eIF2α in the ASI neuron pair did not result in diminished progeny production or extended egg-laying period, indicating that the reproductive impairment is phosphorylation-status-specific. (Figure S9, note figure legend). In addition, the systemic defects driven by the neuronal phosphomutant eIF2α were significantly suppressed by the *eIF2Bα* and *eIF2γ* mutations that confer insensitivity to eIF2α phosphorylation (Figure S10). We note that there is a threshold for the degree of translational inhibition to elicit the organismal response, as reduction of the phosphomutant gene dosage in the heterozygote or by RNAi-mediated transgene silencing did not fully trigger the phenotype (data not shown).

We note that genetic ablation of the ASI neurons, unlike constitutive eIF2α phosphorylation, had no effect on non-dauer growth and reproduction (Figure S9), suggesting that alterations in ASI proteome or signaling mechanisms—rather than general neuronal toxicity or death—are responsible for these cell-non-autonomous

phenotypes in response to changes in neuronal translational status. Because constitutive eIF2α phosphorylation in the ASI neurons elicited a starvation-like response, it is plausible that key molecular regulators of nutritional stress adaptations are responsible for the phenotype. SKN-1/NRF2, which functions in the ASI neurons to regulate lifespan extension upon dietary restriction (Bishop and Guarente, 2007), was not required for the reproductive defects induced by neuronal phosphomimetic eIF2α (Figure S11). Mutation in *daf-12* which encodes a nuclear hormone receptor the activity of which is required for proper growth or diapause decision in response to nutritional availability, was unable to suppress the phenotype triggered by ASI-specific eIF2α phosphorylation (Figure S11). The absence of DAF-18/PTEN activity, which would result in cytoplasmic sequestration of DAF-16/FOXO that regulates stress resistance, also had no effect on the organismal defects caused by ectopic neuronal eIF2α phosphorylation (Figure S11). We note that during larval development overexpression of ASI-specific eIF2(αP) was able to only modestly enhance dauer entry in the *daf-2* insulin receptor mutant (Figure 4A), suggesting that neuronal eIF2α phosphorylation may act in part through insulin signaling at multiple life stages, albeit independently of PI3K signaling during adulthood (Figure S11).

Discussion

We set out to genetically characterize the organismal effects of cell-specific eIF2α phosphorylation, utilizing the mutant DAF-28 insulin that induces neuron-specific phosphorylation of eIF2α to promote dauer diapause. Dauer formation is a physiological stress response and a rapidly identifiable developmental phenotype, suitable for a forward

genetic approach. Our genetic analysis has revealed neuronal translation control, mediated by conserved translational regulatory factors, as a novel pathway governing the organismal diapause decision during larval development, as well as metabolism and reproduction in adulthood.

The isolation of multiple alleles of genes characterized in our previous study (Kulalert and Kim, 2013), and of those modulating ER client protein folding, confirms the specificity of the screen and indicates saturation. The recovery of the loss-of-function and revertant alleles of *daf-28* further underscores insulin misfolding as a source of ER homeostasis perturbation, and suggests proteotoxic contribution of unpaired cysteine, as in the case of the Akita diabetes mouse model (Wang et al, 1999). Our genetic analysis of the *C. elegans* ERp44 also points to a conserved function of the isomerase in modulating maturation of insulin (Pottekat et al., 2013 and Yang et al., 2016). A multitude of *pek-1* loss-of-function alleles isolated in this screen could also provide resources for structural and functional studies of the critical residues or domains of the conserved eIF2α kinase, particularly in the organismal context.

Identification of an organismal role of the C. elegans translation regulatory factor functioning downstream of neuronal eIF2α phosphorylation

eIF2B is a heterodecameric complex that plays a critical role in regulating translation initiation (Kashiwagi et al., 2016). The biochemical nature of eIF2B and its regulation has been characterized at the cellular level in yeast, flies and mammals (Hinnebusch 1997 and Williams et al., 2000). Of note, genetic analysis of eIF2B subunits has not been performed in the systemic, cell nonautonomous context in multicellular

organisms such as *C. elegans*. In this study, we have shown that mutations in the worm *eIF2Bα* resulted in insensitivity to eIF2α phosphorylation at the developmental decision level, in addition to the cellular level previously characterized in yeast (Yang and Hinnebusch, 1996). The *eIF2Bα* alleles isolated behaved similarly to loss of function of *eIF2Bα,* consistent with the dispensable, regulatory role of GCN3 in *S. cerevisiae*. The dominant nature of the *eIF2Bα* deletion allele in suppressing the organismal response to eIF2α phosphorylation suggests that eIF2B susceptibility to eIF2(αP) is readily perturbed by reduced eIF2Bα dosage. The *eIF2Bα* mutations that confer the dominant suppressor phenotype similar to the loss-of-function allele likely affect residues that are critical for eIF2Bα functional and structural integrity. Additionally, because eIF2Bα forms homodimer as part of the eIF2B holoenzyme, the altered eIF2Bα may exert a dominant-negative effect that prevents proper dimerization or holoenzyme formation (Kashiwagi et al., 2016).

We note that four *eIF2Bα* alleles alter conserved residues at equivalent positions and domains to those in the other regulatory subunits of eIF2B, eIF2Bβ and eIF2Bδ (Figure S4), reminiscent of the yeast eIF2(αP)-insensitive mutations affecting homologous residues in all three regulatory eIF2B subunits (Pavitt et al., 1997). While eleven *eIF2Bα* alleles were recovered in our screen, alleles of genes encoding eIF2Bβ and eIF2Bδ were not isolated. The beta and delta subunits, unlike the alpha subunit, are indispensable for *C. elegans* development (Figure 3B). It is thus likely that partial loss of function of eIF2Bβ and eIF2Bδ is not tolerable, or that eIF2Bα may play a prominent role in translational control in the chemosensory neural network.

In light of our genetic analysis of eIF2B in mediating translational control in the *C. elegans* chemosensory neurons, we note that more than 120 mutations in genes encoding human eIF2B subunits have been associated with vanishing white matter disease, which involves hypomyelination in the central nervous system (reviewed in Bugiani et al., 2010). The enrichment of neuropathology-associated eIF2B mutant alleles suggests critical roles of eIF2B subunits in regulating protein synthesis and homoeostasis in nerve and glial cells in the mammalian brain. We also note that eIF2B regulatory subunit delta has been identified as the molecular target of the drug ISRIB that renders mammalian neurons refractory to eIF2α phosphorylation to enhance memory formation (Sidrauski et al., 2015 and Sekine at al., 2015). These findings illustrate critical roles of eIF2B-mediated translational control in the nervous system of *C. elegans*, as well as of higher animals.

Characterization of a novel mutation in the C. elegans eIF2 subunit that confers systemic insensitivity to neuronal eIF2α phosphorylation

The genetic screen also allowed us to study the function of the eIF2 gamma subunit at the organismal level. The alteration in the conserved Ser443 of eIF2γ confers insensitivity to eIF2α phosphorylation through a mechanism distinct from the recovered *eIF2Bα* mutants that result in removal of the binding site of eIF2(αP). Specifically, the S443L change in eIF2γ results in compensatory bypassing of an essential function of eIF2B, rendering the eIF2Bγ subunit dispensable, whereas *eIF2Bα* mutation, as well as *eIF2α* phosphorylation site mutation, did not (Figures 3B and S7).

The functional consequences of the *eIF2γ(S443L)* mutation are also distinct from most of the previously characterized mutations in the yeast eIF2 subunits, which result in altered start codon recognition (Donahue et al., 1988 Alone et al., 2008 and Cigan et al., 1989) and global defects in protein synthesis and growth (Erickson and Hannig, 1996 and Borck et al., 2012). We did not observe impaired development and reproduction that are symptomatic of general translational attenuation in the *C. elegans eIF2γ* mutant. Similar to the *eIF2γ* mutation in our study, a few previously studied eIF2 subunit alterations specifically abrogate eIF2(αP) sensitivity without inhibiting global protein synthesis. For instance, mutations in the yeast *eIF2α* that weaken interactions between eIF2α and eIF2α kinases or between eIF2(αP) and eIF2B diminish sensitivity to translational control by eIF2α phosphorylation, thus compromising survival specifically under amino acid starvation that activates eIF2α kinases (Vazquez de Aldana et al., 1993 and Dey et al., 2005). Notably, a recent study identified a mutation in *S. cerevisiae eIF2β* that does not attenuate general translation but reduces sensitivity to eIF2(αP) under stress (Jennings et al., 2016). The *eIF2β* mutant circumvents translational control by eIF2(αP) via a distinct mechanism from the *C. elegans eIF2γ(S443L)* mutation, as the yeast *eIF2β* mutant is able to bypass the requirement for eIF2Bε, whereas the *C. elegans eIF2γ* mutant still depends on eIF2Bε for viability (Figure 3B and Jennings et al., 2016).

We note that the *C. elegans* eIF2γ(S443L) alteration represents a novel class of eIF2γ mutation. Unlike the eIF2γ alterations described in Erickson and Hannig, 1996, Alone et al., 2008 and Borck et al., 2012, which impair cell growth, the *eIF2γ(S443L)* mutation does not manifest developmental or reproductive impairments. Of note, the

yeast *GCD11/eIF2γ(K250R)* mutation does undermine growth, but also bypasses translational control by eIF2α phosphorylation (Erickson et al., 2001). The *C. elegans eIF2γ(S443L)* mutation contributes to eIF2(αP) insensitivity via a different mechanism from the yeast *GCD11/eIF2γ(K250R)*. Whereas the *GCD11(K250R)* mutation obviates the requirement for essential functions of eIF2α and all eIF2B subunits, the S443L alteration in eIF2γ was able to functionally substitute only for eIF2Bγ (Figure 3B). Importantly, the S443L substitution did not alter the requirement for eIF2Bε, which is the key subunit catalyzing the GDP-GTP exchange, suggesting that the mutation does not merely augment guanine nucleotide dissociation, as in the case of the *gcd11(K250R)* mutant. Both Lys250 and Ser443 are highly conserved residues of eIF2γ, but they reside in two distinct domains (Domains II and III, respectively), which may differentially influence eIF2γ affinity to GTP/GDP and interaction with other translation regulatory factors.

Our mechanistic interpretations and distinctions among different eIF2 subunit mutants are mainly derived from the extensive genetic and biochemical studies conducted in yeast, in light of the high conservation of residues and domains across eukaryotes. Our *C. elegans* genetic characterization of the novel *eIF2γ(S443L)* mutant unambiguously demonstrates that a subset of eIF2B catalytic functions is dispensable for translation initiation and organismal viability, enabling a unique mechanism to bypass translational regulation by eIF2α phosphorylation (Figure 3).

Aberrant neuronal phosphorylation status impacts the organismal physiology at multiple life stages

We have shown that ASI-specific eIF2α phosphorylation is able to promote dauer formation independently of activation of the UPR/PEK-1 described in our previous study (Figure 4, in comparison to Kulalert and Kim, 2013), consistent with the central role of phosphorylation of eIF2α in integrating multiple adverse cues that include not only ER stress but also nutritional deficit (Rousakis et al., 2013). The ability of the neuronal phosphomutant eIF2α to trigger dauer entry in the wild-type and TGFβ-deficient backgrounds (Figure 4A), in addition to in the presence of misfolded neuropeptide (Kulalert and Kim, 2013), suggests a developmental role of neuron-specific eIF2α phosphorylation in a broader or more physiological context. Chapter IV will further discuss how ASI-specific eIF2α phosphorylation may mimic the pro-dauer effects elicited by heat-killed bacteria that have key modulatory roles in dauer formation in the presence of the ascarosides.

Intriguingly, ectopic neuronal eIF2(αP) modestly enhanced entry into dauer diapause in the *daf-2* insulin receptor mutant background, suggesting that neuronal eIF2α phosphorylation may function partially through insulin signaling mechanism, while largely functioning in parallel to TGFβ and population density-sensing pheromone pathways. Further experiments will be required to address whether constitutive eIF2α phosphorylation may alter translation of various insulin peptides secreted from the ASI neurons, contributing to the dauer decision.

The ASI neurons play a central neuroendocrine role in the regulation of *C. elegans* physiology, modulating dauer formation, satiety quiescence and longevity in response to alteration in nutritional status (Bargmann and Horvitz, 1991, Ren et al., 1996, Schackwitz et al., 1996, Alcedo and Kenyon, 2004, Bishop and Guarente, 2007,

Gallagher et al., 2013). The neurosecretory demand and the nutrient-sensing function of the neurons may be coupled to translational control mechanism such as eIF2α phosphorylation. We have previously shown that the ASI neuron pair was susceptible to perturbations in ER protein-folding homeostasis and that the eIF2α kinase PEK-1 can be readily activated in the neurons to promote stress adaptations (Kulalert and Kim, 2013). Food-dependent translational control in the nervous system has been studied in invertebrates such as *Drosophila*, in which nutritional signals modulate transcription of *4eBP*, resulting in attenuation of protein synthesis (Mahoney et al., 2016). In *C. elegans*, the amino acid-sensing eIF2α kinase GCN-2 functions in starvation response (Rousakis et al., 2013), but the site of action remains elusive. It is conceivable that the ability of the ASI neuron pair to perceive nutrient deprivation may in part rely on GCN-2 activity, such as detection of uncharged tRNAs, and resultant translation attenuation. The reduction in brood size in response to constitutive eIF2α phosphorylation in ASI is also consistent with previous studies that associate impaired progeny production with limited food availability. Furthermore, the DAF-7/TGFβ ligand produced by the ASI chemosensory neurons has been shown to function to couple germline development to chemosensory experience (Dalfo et al., 2012). In addition to reduction in progeny production, neuronal eIF2(αP) results in clear appearance and extended reproduction, reminiscent of the phenotypes exhibited by the *eat* mutants that are defective in food intake (Avery 1993, Lakowski and Hekimi, 1998 and Figure S8).

Dysregulation of translation initiation in the neurons can have both cell-autonomous and cell-nonautonomous consequences underlying organismal pathologies. For instance, unabated stressors such as prion infection diminish translation in an

eIF2(αP)-dependent manner, resulting in neuronal death (Moreno et al., 2012). Aberrant phosphorylation of eIF2α in the immune and nervous systems can also cell nonautonomously impair tissue homeostasis and complex behaviors (Hao et al., 2005, Costa-Mattioli et al., 2007, Stern et al., 2013 and Ravindran et al., 2016). In *C. elegans*, we have shown that neuronal eIF2(αP) elicits a starvation-like response that is distinct from the apparent normal growth and reproduction phenotype upon neuronal ablation, indicating that, instead of triggering cell death, constitutive eIF2α phosphorylation alters the ASI translatome in a manner that results in net neural or secretory output that induces adaptive metabolic remodeling. Our study indicates that translational status in the chemosensory neurons, which may be modulated by environmental and developmental cues, contributes to organismal stress responses at both larval and adult stages. Stress-induced eIF2α phosphorylation in the nervous system therefore plays a critical role in inter-tissue communication and coordination, regulating multiple aspects of animal physiology in *C. elegans*.

Experimental procedures

Only experimental procedures that have not been described in Chapter II are listed here.

Suppressor mutant isolation and characterization

Mutagenesis using ethyl methanesulfonate (EMS) was performed on the starting strain carrying the *daf-28(sa191)* allele. F2 generation eggs from sixteen independent F1 generation pools were plated onto NGM plates seeded with *E.Coli* strain OP50. The plates were screened for animals that failed to enter dauer diapause 48 hours later at

25°C. Specifically, we isolated L4 or young adult F2 animals. The recovered mutants were retested and selected based on viability and the ability to give rise to largely non-dauer population in subsequent generations.

We then crossed the mutants into the starting strain to determine mode of inheritance. For recessive suppressor mutants, complementation testing was performed using previously characterized suppressors such as *daf-28(sa191);pek-1(ok275)*, as well as among mutants from distinct pools for complementation group assignment. Representative mutants were outcrossed at least three times to the starting strain and were submitted for whole genome resequencing and bioinformatics analyses, performed by BGI Americas. We then identified nucleotide polymorphisms that were unique to each mutant, and confirmed whether the candidate mutations were causative of the phenotypic suppression using previously characterized deletion alleles.

Constructs and generation of transgenic animals

The promoter of the *gpa-4* gene was used as an ASI-specific promoter to express transgenes (Jansen et al., 1999, Bishop and Guarente, 2007). Importantly, the *gpa-4* promoter was able to drive heterologous gene expression to mediate organismal phenotypes in both larval and adult stages, specifically dauer formation and diet-restriction-mediated lifespan extension (Bishop and Guarente, 2007, Kulalert and Kim, 2013).

Gamma radiation was employed to integrate the *qdIs10[gpa-4p::eIF2α(S49D)]* extrachromosomal arrays. The animals carrying the integrated arrays were then

outcrossed nine times. Animals carrying extrachromosomal or integrated arrays both exhibited similar starvation-like phenotypes.

Pheromone-induced dauer formation assay

Unless otherwise noted, all dauer formation assays were done without the dauer pheromone, as described in Kulalert and Kim, 2013. In Figures 4 and S6, 100 μL of the pheromone mix consisting of synthetic ascarosides #2, #3, #5 and #8, provided by Frank Schroeder, was added onto 3.5 cm plates made with Noble agar and without peptone. Concentrated heat-killed or live *E. coli* OP50 were then seeded onto the plates. Egg laying was done at 25°C using around three adults of indicated genotypes for 3-5 hours, and scoring took place 72 hours afterward. The average total number of animals per plate was approximately 40 to 60.

Brood size assay

Individual L4 animals were placed onto NGM plates seeded with *E. coli* strain OP50 at 25°C. Each animal was then transferred to individual plates on a daily basis for at least five days. The number of eggs laid or progeny hatched was then scored every day. The results shown were based on multiple animals from at least two independent experiments.

RNAi of translation initiation factor genes

RNAi by bacterial feeding using *E. coli* HT115 bacteria was carried out as reported. All vectors used in this study were validated by Sanger sequencing. For each

experiment, bacteria expressing the empty L4440 vector (negative control RNAi), the L4440-derived *unc-22* RNAi vector (positive control RNAi, based on the twitching phenotype induced by *unc-22* knockdown) and the respective L440-derived translation regulatory factory gene RNAi vectors were included. L4 animals were fed on RNAi bacteria plates for 72 hours at 16°C, and the F_1 generation animals were scored for fractions that reached L4 and subsequent fertile adulthood at 25°C. Results shown were based on multiple independent experiments.

Microscopy

For lipid storage visualization, Sudan Black staining was performed, using a protocol adapted from Kimura et al., 1997. Specifically, animals were fixed in 1% formaldehyde, stained with 50% Sudan Black solution and washed in M9 buffer four times before mounting.

Figure Legends

Figure 1: Identification of genes required for the larval organismal response to neuronal UPR activation and subsequent eIF2α phosphorylation

A. Schematic illustrating the alleles and gene products characterized in the study.
B. Table listing the identified genes, their respective mammalian orthologs, numbers of alleles recovered, and fractions of dauers and total number of animals for indicated suppressor genotypes.
C. Fluorescence microscopy of the animals with indicated genotypes carrying the *hsp-4p::GFP* (*zcIs4*) transgene (GFP).

Figure 2: eif-2b.1 encodes the C. elegans ortholog of the alpha subunit of eIF2B that mediates cellular and organismal sensitivity to eIF2α phosphorylation

A. Table listing fractions of dauers and total number of animals for indicated suppressor genotypes.

B. Fluorescence microscopy of the animals with indicated genotypes carrying the transgene (GFP) driven under the promoter and uORF of *atf-5*. Arrowheads mark the spermatheca.

Figure 3: The eif-2.3(qd336) allele, encoding a mutant C. elegans ortholog of the gamma subunit of eIF2, alters sensitivity to eIF2α phosphorylation and translational requirements of eIF2B

A. Schematic illustrating the *qd336* variant of eIF-2.3 and its alteration of function in the context of translation initiation.

B. Fractions of the animals with indicated genotypes that reach reproductive maturation under RNAi conditions that downregulate translation regulatory factors. Plotted is mean ± SD. N represents total number of animals for each condition.

C. Fluorescence microscopy of the animals with indicated genotypes carrying the transgene (GFP) driven under the promoter and uORF of *atf-5*. Arrowheads mark the spermatheca.

Figure 4: Aberrant eIF2α phosphorylation in the ASI chemosensory neurons modulate the dauer developmental decision in response to neuroendocrine and environmental signals

A. Fractions of the animals with indicated genotypes that form dauer at 25 °C. Plotted is mean ± SD. N represents total number of animals for each genotype. Results shown here are representative of three independent experiments.
B. Fractions of the animals with indicated genotypes that form dauer at 25 °C, in the presence of ascarosides and live bacteria. Plotted is mean ± SD. Results shown here are based on two experiments, representative of five independent experiments.

Figure 5: Constitutive eIF2α phosphorylation in ASI impairs growth, metabolism and reproduction, phenocopying the organismal responses to starvation

A. Bright-field imaging of age-matched adult animals with indicated genotypes.
B. Body length and width measurements, based on 10 representative animals from each indicated genotype. Plotted is mean ± SD
C. Growth rate of animals with indicated genotypes.
D. Lipid staining of age-matched adult animals with indicated genotypes.
E. Total number of progeny production of the animals with indicated genotypes. Plotted is mean ± SD
F. Egg-laying period of the animals with indicated genotypes. Plotted is mean ± SD

G. Number of fertilized embryos observed in the hermaphrodite animals of indicated genotypes. Plotted is mean ± SD

H. Fluorescence microscopy of the animals with indicated genotypes carrying the transgene (GFP) driven under the promoter of the lipase gene *atgl-1*. The co-transformation marker of *qdIs10* also expresses GFP in the coelomocytes.

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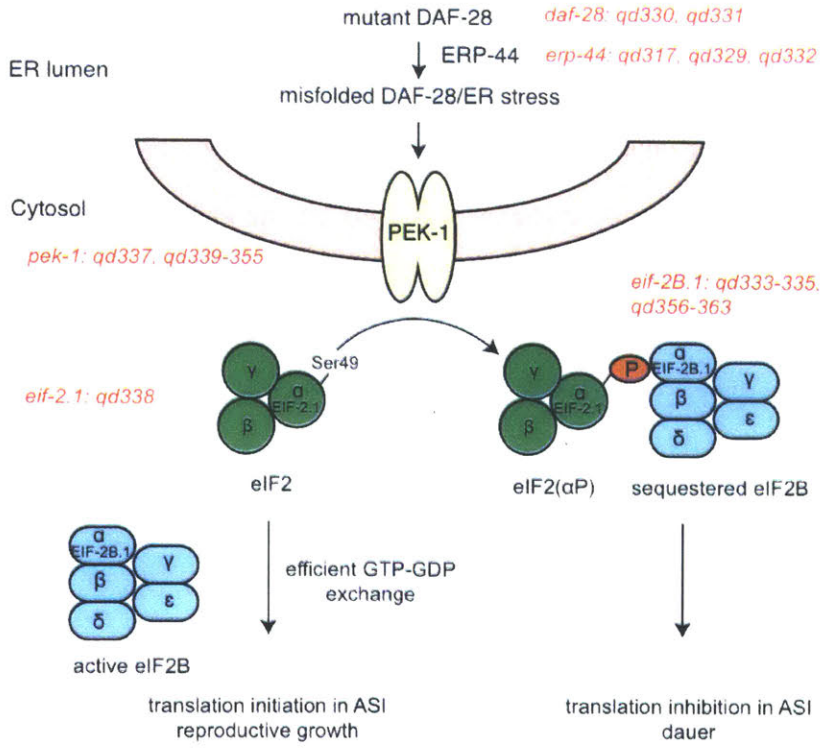
A



B

Gene	Mammalian ortholog	Number of recovered alleles	Suppressor genotype	Fraction of dauers (mean ± SD)	Total number of animals
daf-28	β-type insulin	2	*daf-28(sa191)**	0.99 ± 0.02	465
			daf-28 (sa191qd330)	0	228
			daf-28 (sa191qd331)	0	214
erp-44 (c30h7.2)	ERp44	3	*erp-44(qd317);daf-28(sa191)*	0.005 ± 0.012	433
			erp-44(qd329);daf-28(sa191)	0.026 ± 0.031	347
pek-1	PERK	18	*daf-28(sa191);pek-1(qd337)*	0	231
			daf-28(sa191);pek-1(qd342)	0	210
eif-2.1 (y37e3.10)	eIF2α	1	*eif-2.1(qd338);daf-28(sa191)*	0	131
eif-2B.1 (zk1098.4)	eIF2Bα	11	*eif-2B.1(qd333);daf-28(sa191)*	0.002 ± 0.003	580
			eif-2B.1(qd334);daf-28(sa191)	0.01 ± 0.01	472
eif-2.3 (y39g10ar.8)	eIF2γ	1	*eif-2.3(qd336);daf-28(sa191)*	0	119

C

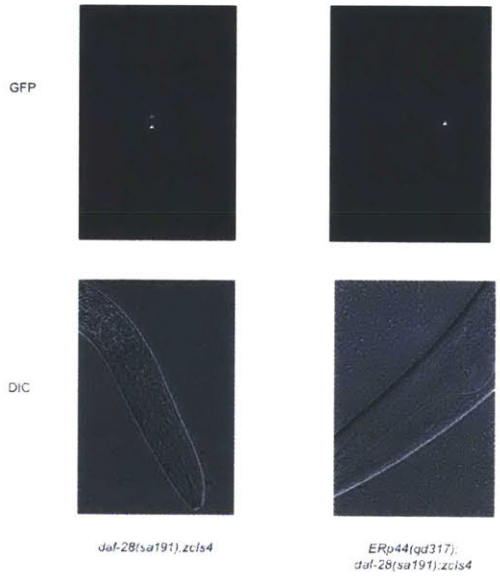


Figure 2

A

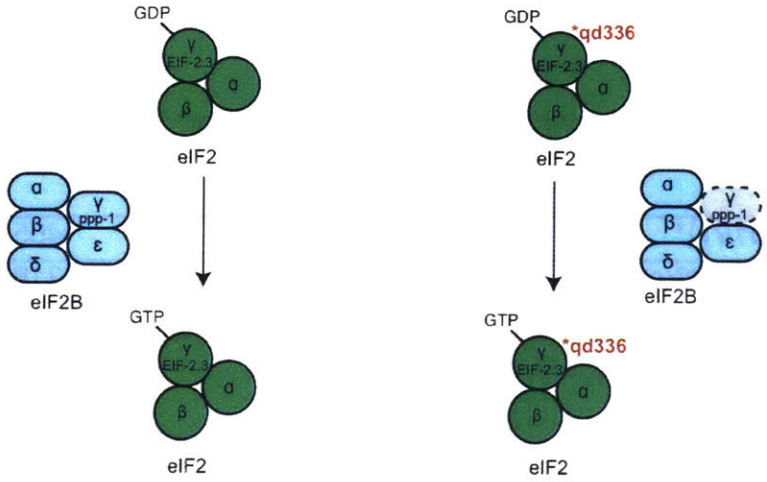
Suppressor genotype	Fraction of dauers (mean ± SD)	Total number of animals
*daf-28(sa191)**	0.99 ± 0.02	465
eif-2B.1(qd335);daf-28(sa191)	0.06 ± 0.006	318
eif-2B.1(pk720);daf-28(sa191)	0.01 ± 0.02	133

B

wild-type *eif-2.1(qd338)* *eif-2B.1(qd333)*

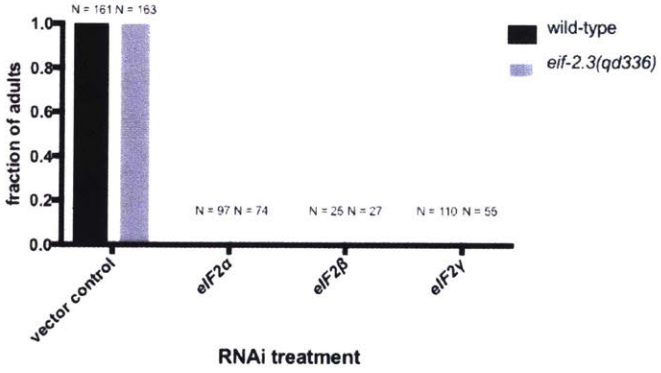
Figure 3

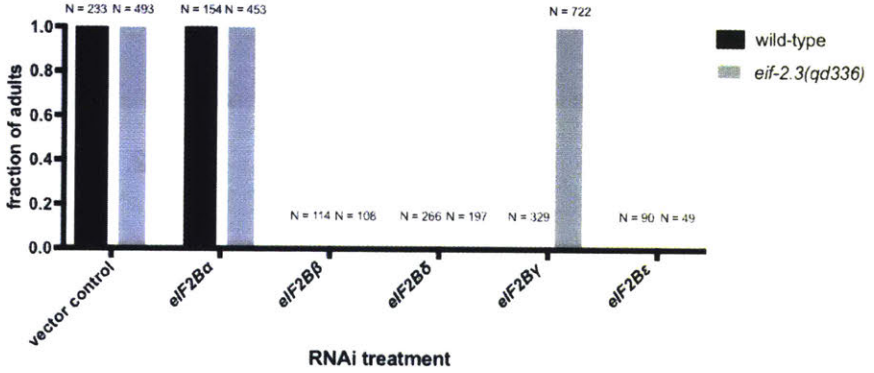
A



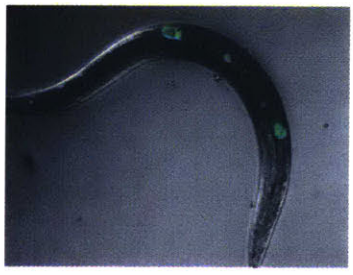
translation initiation
devlopmental viability

B





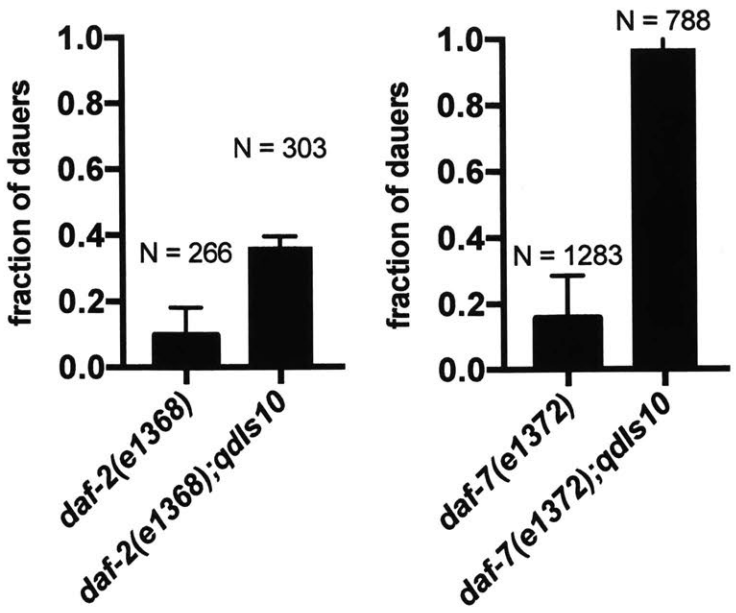
C



wild-type

eif-2.3(qd336)

A


fraction of dauers
1.0
0.8
0.6
0.4
0.2
0.0
N = 303
N = 266
daf-2(e1368)
daf-2(e1368);qdls10
N = 788
N = 1283
daf-7(e1372)
daf-7(e1372);qdls10

B

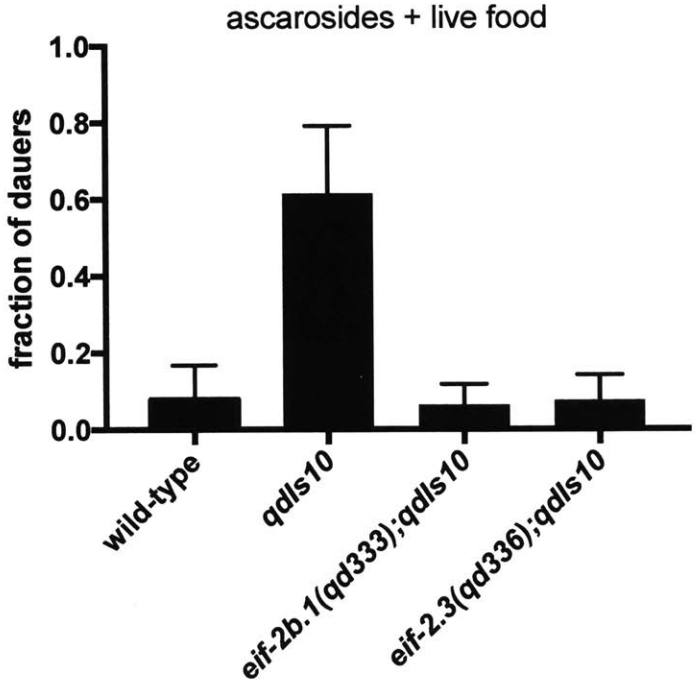
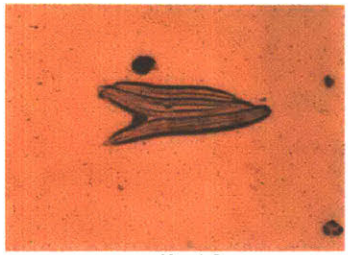

ascarosides + live food
fraction of dauers
1.0
0.8
0.6
0.4
0.2
0.0
wild-type
qdls10
eif-2b.1(qd333);qdls10
eif-2.3(qd336);qdls10

Figure 5

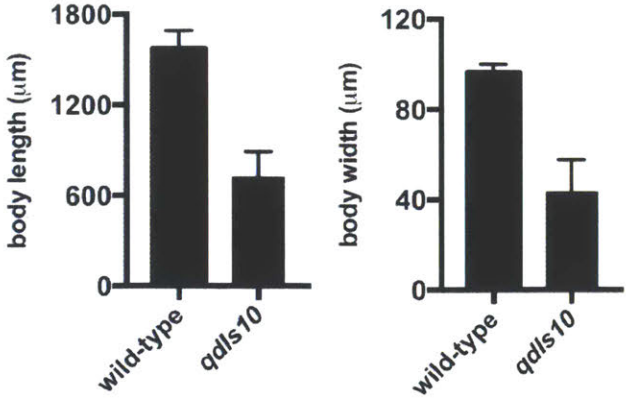
A

wild-type

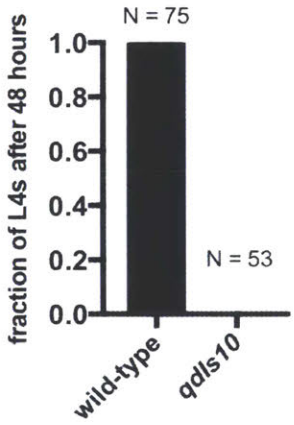


qdIs10

B

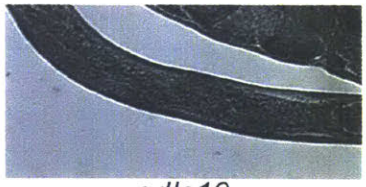


C



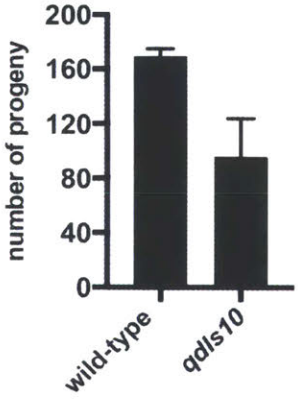
D

wild-type

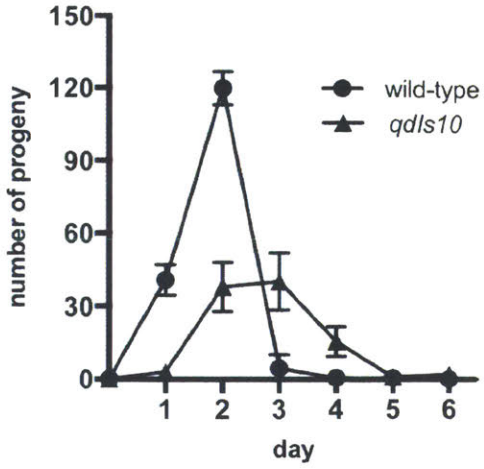


qdIs10

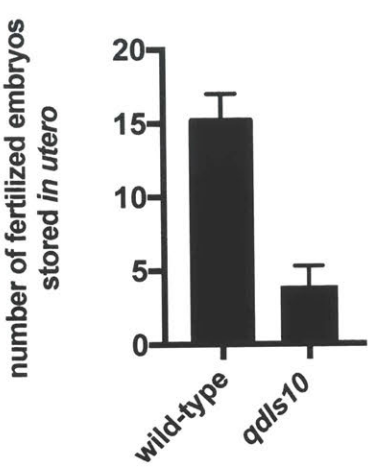
E



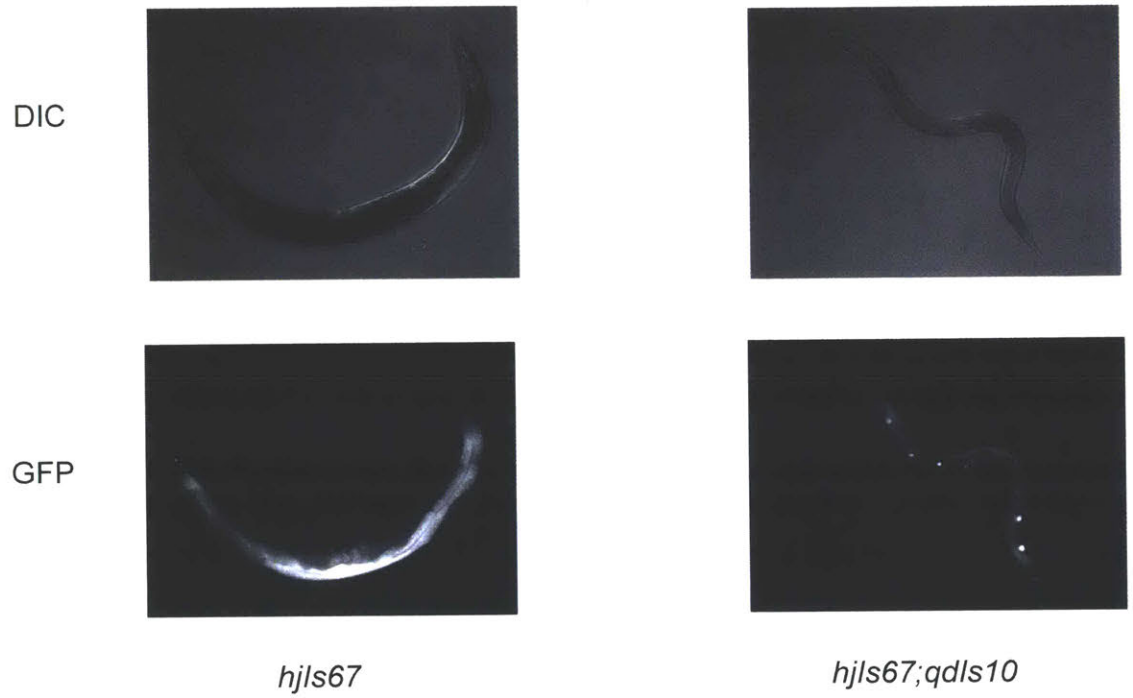
F



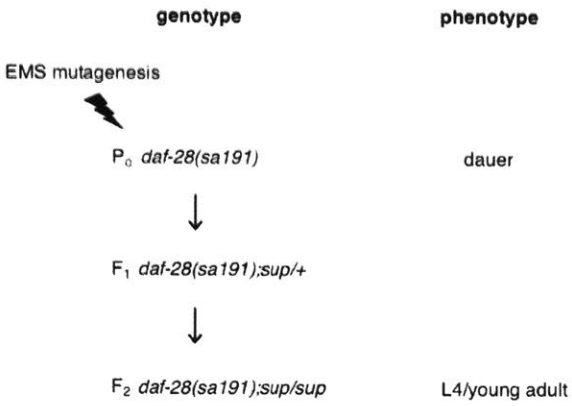
G


number of fertilized embryos stored *in utero*
20
15
10
5
0
wild-type
qdls10

H


DIC
GFP
hjls67
hjls67;qdls10

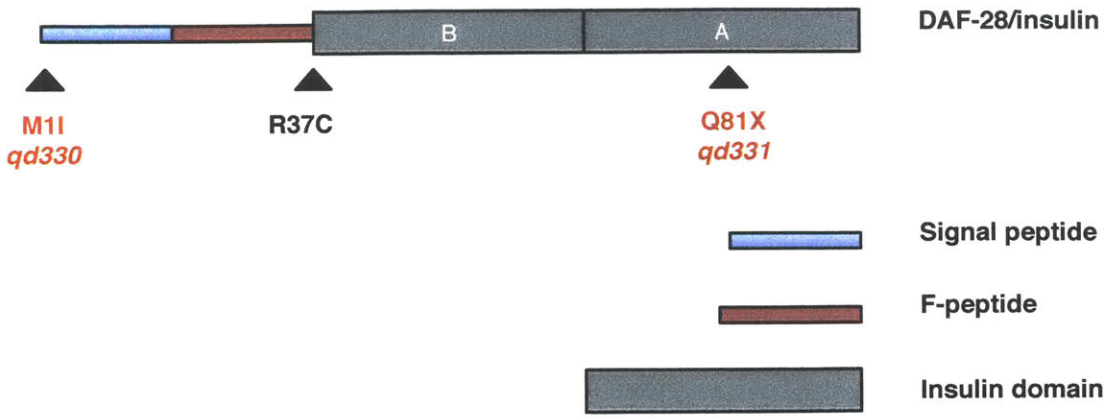
A



B

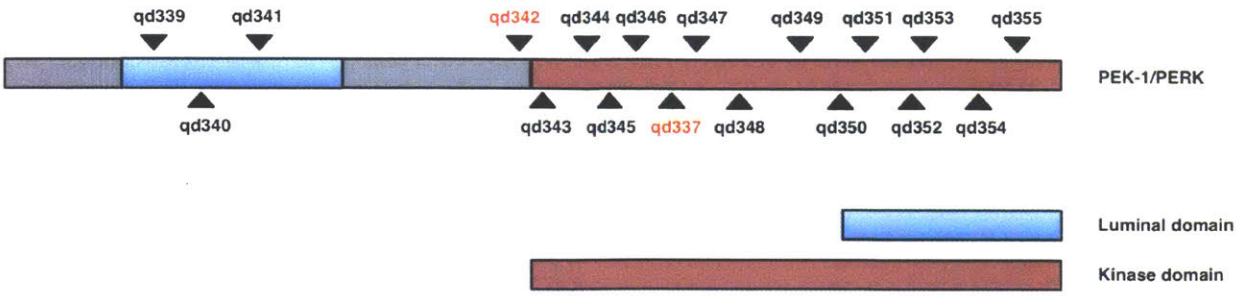
Gene	Allele	Molecular identity
daf-28	*sa191qd330*	M1I R37C
	sa191qd331	R37C Q81X
erp-44	*qd329*	W4X
	qd317	splice site (G to A), 3′ to exon 2
	qd332	Q213X
pek-1	*qd339*	G63E
	qd340	G147E
	qd341	P236L
	qd342	R519X
	qd343	G613D
	qd344	G616D
	qd345	D626N
	qd346	A631T
	qd337	R649X
	qd347	L654F
	qd348	E670K
	qd349	G875D
	qd350	G937R
	qd351	D956N
	qd352	G986S
	qd353	P993S
	qd354	G1037E
	qd355	splice site (G to A), 5′ to exon 12
eif-2.1	*qd338*	S49F
eif-2b.1	*qd333*	E28K
	qd334	T41I
	qd356	A70V
	qd357	R98C
	qd358	H132Y
	qd359	G200C
	qd360	G208D
	qd361	G215E
	qd362	S225L
	qd363	D274N
	qd335	M1*
eif-2.3	*qd336*	S443L

Figure S1: Suppressor mutations identified from the *daf-28(sa191)* constitutive dauer entry phenotype
A. Schematic detailing the forward genetic approach used to isolate the F2 mutants that failed to enter dauer diapause constitutively under laboratory conditions.
B. List of altered residues or splice sites caused by the suppresor mutations. Amino acid positions and splice sites are based on the c30h7.2a.1 isoform of erp-44. The qd335 allele results in a new start codon just a few base pairs upstream of the original AUG, likely resulting in early translation initiation of an out-of-frame ORF



Genotype	Phenotype
daf-28(R37C)/daf-28(R37C)	dauer
daf-28(M1I R37C)/daf-28(M1I R37C)	L4
daf-28(M1I R37C)/daf-28(R37C)	dauer
daf-28(-)/daf-28(R37C)	dauer
daf-28(R37C Q81X)/ daf-28(R37C Q81X)	L4
daf-28(R37C Q81X)/daf-28(R37C)	L4
daf-28(+)/daf-28(R37C)	L4

Figure S2: Second-site mutations in *daf-28* suppress the dauer entry phenotype
We identified a second-site mutation in the start codon (M1I) that resulted in intragenic suppression of the constitutive dauer entry phenotype. Consistent with its loss-of-function alteration, the *daf-28(M1I R37C)* allele behaved similarly to the *daf-28* null allele, specifically in heteroallelic combination with *daf-28(R37C)*. We also isolated a second-site nonsense mutation (Q81X) that behaved similarly to the *daf-28* wild-type allele in heteroallelic combination with *daf-28(R37C)*, suggesting revertant alteration. The "dauer" phenotype refers to frequency of dauer formation higher than 90%. The "L4" phenotype refers to frequency of dauer formation lower than 10%. The phenotypes were based on at least three trials, with at least 100 animals per genotype. Data for *daf-28(-)/ daf-28(R37C)* and *daf-28(+)/daf-28(R37C)* were presented in Kulalert and Kim, 2013. Schematic above the table represents protein domains of DAF-28 and was adapted from Li et al., 2003.



Allele of *pek-1*	Molecular identity
qd339	G63E
qd340	G147E
qd341	P236L
qd342	R519X
qd343	G613D
qd344	G616D
qd345	D626N
qd346	A631T
qd337	R649X
qd347	L654F
qd348	E670K
qd349	G875D
qd350	G937R
qd351	D956N
qd352	G986S
qd353	P993S
qd354	G1037E
qd355	splice site (G to A), 5′ to exon 12

Figure S3: Mutations in *pek-1* suppress dauer entry triggered by neuronal ER stress
All *pek-1* mutations listed resulted in suppression of the dauer entry phenotype to a similar degree to that illustrated in Figure 1C for *pek-1(qd337)*. Schematic above is based on conserved domains. X (table) and red font (schematic) represent stop codon and nonsense mutatation respectively.

Allele of *eIF2Bα*	Molecular identity	Mode of inheritance	Residue conservation
pk720	insertion/deletion	dominant	N/A
qd333	E28K	dominant	variable
qd334	T41I	dominant	conserved
qd356	A70V	dominant	variable
qd357	R98C	recessive	variable
qd358	H132Y	dominant	conserved
qd359	G200C	dominant	conserved*
qd360	G208D	dominant	conserved*
qd361	G215E	dominant	conserved*
qd362	S225L	recessive	variable
qd363	D274N	dominant	conserved*
qd335	out-of-frame upstream ATG	recessive	N/A

Figure S4: Mode of inheritance of the constitutive dauer entry suppression conferred by different alleles of *C. elegans eIF2Bα*

We determined the phenotypic mode of inheritance by crossing the isolated suppressor strain (*daf-28(sa191);sup/sup*) to the starting strain (*daf-28(sa191);+/+*). If the F1 progeny (*daf-28(sa191);sup/+*) does not form dauers constitutively (i.e. one copy of *sup* is able to suppress the dauer entry phenotype), the allele is considered to behave dominantly. If the F1 progeny forms dauer constitutively, the allele contributes to a recessive supression phenotype.

Residue conservation was based on yeast, worm, fly rat, mouse and human eIF2Bα amino acid sequences, using BLOSOM as previously analysed in Williams et al., 2001 and other eIF2B studies. The asterik denotes residue conservation with both eIF2Bα orthologs and other regulatory subunits of eIF2B, eIF2Bβ and eIF2Bδ.

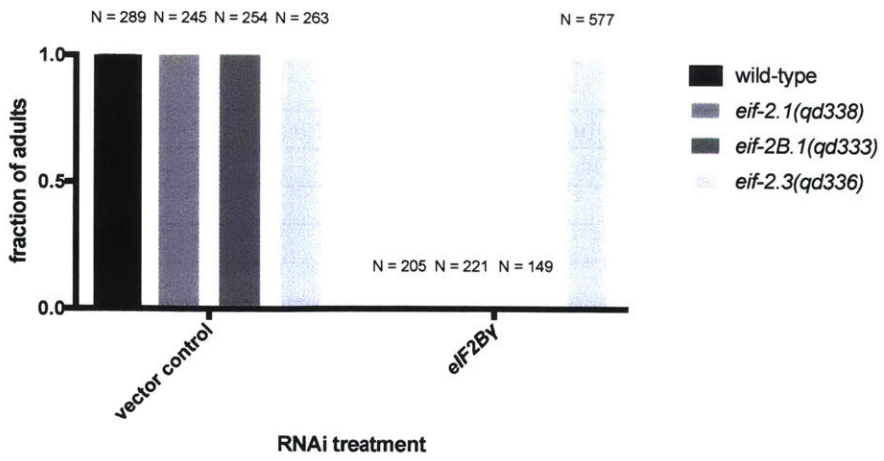


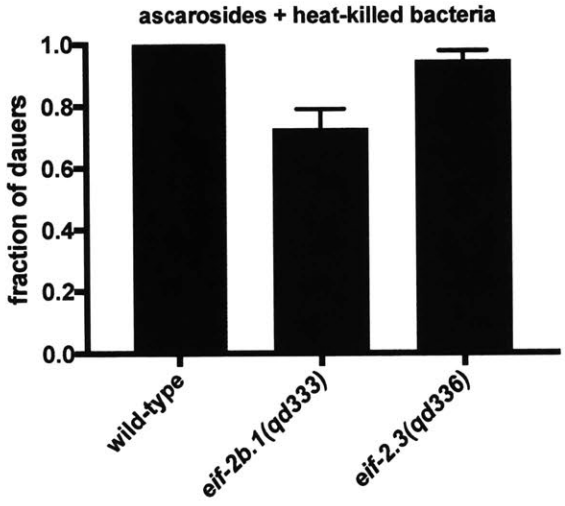
Figure S5: Effects of RNAi knockdown of *eIF2Bγ* on larval development of on various eIF2(α P)-insensitive mutants

The *qd338* allele alters the Ser49 phosphorylation site of eIF2α.The *qd333* allele behaves similarly to the *eIF2Bα* loss-of-function mutation. The *qd336* allele results in S443L alteration in eIF2γ.

A

Genotype	Dauers on starved-out plates?
wild-type	Yes
eif-2.1(qd338)	Yes
eif-2b.1(qd333)	Yes
eif-2.3(qd336)	Yes
daf-12(m20)	No

B



C

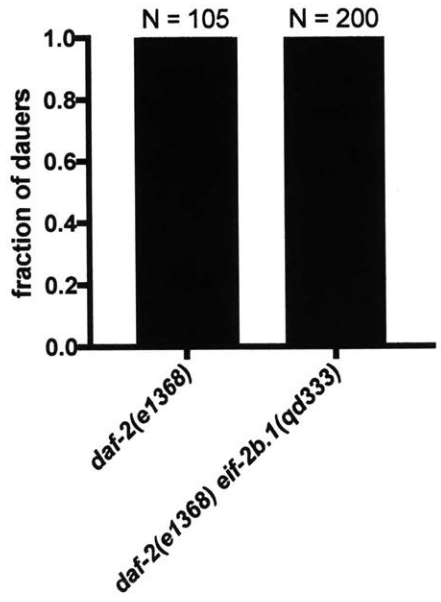


Figure S6: Examination of the roles for eIF2(αP) insensitivity in the dauer developmental decision

A. Multiple starved-out plates of animals with indicated genotypes were examined. The *daf-12* mutant was used as a Daf-d control.

B. The animals with indicated genotypes were grown in the presence of ascarosides and killed bacteria.

C. The animals with indicated genotypes were grown under normal growth conditions.

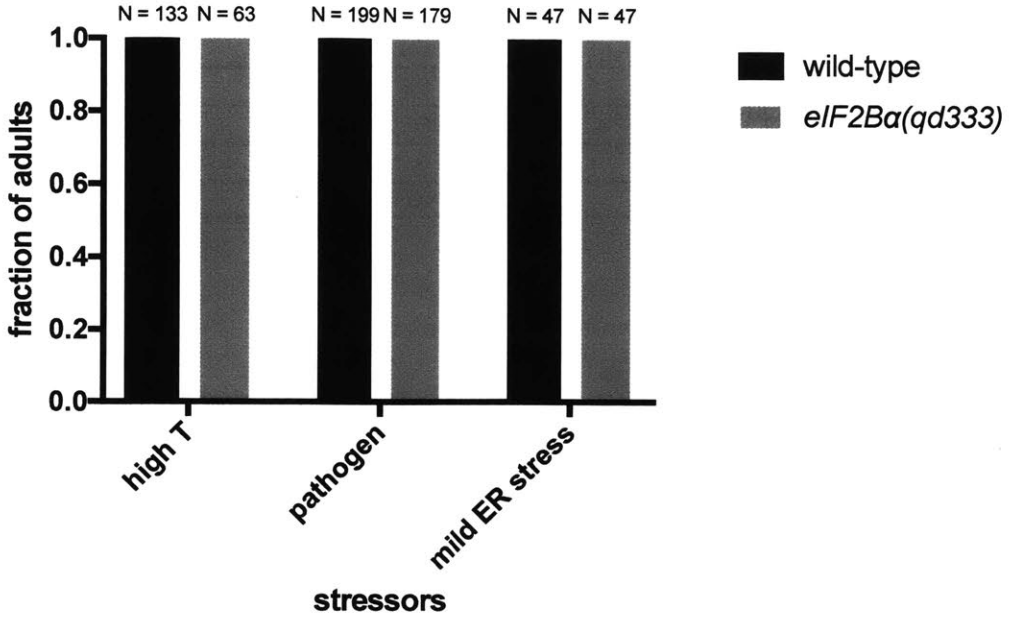


Figure S7: eIF2α-P-insensitivity does not impair development under stressful laboratory conditions

Eggs were laid under unfavorable conditions: high T (26.5 °C), pathogen (*Pseudomonas aeruginosa)* and mild ER stress (the drug tunicamycin at 2.5 μg/ml). No apparent phenotypic differences were observed between the eIF2α-P-insensitive mutant and wild-type control. Similar results were observed at a higher concentration of tunicamycin (4 μg/ml), and with a different eIF2α-P-insensitive mutant, *eIF2γ(qd336).*

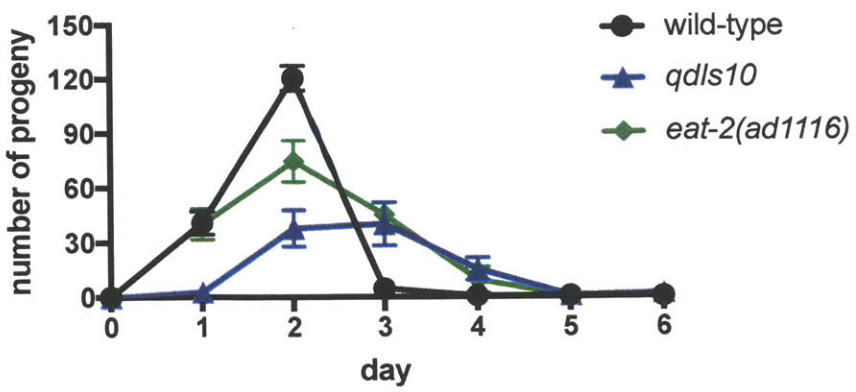


Figure S8: Extension of the egg-laying period in the food uptake-defective *eat-2* mutant, similar to that observed in the *qdIs10* background

The *ad1116* allele results in a splice site mutation in the *eat-2* gene that induces lifespan extension due to nutritional limitation. We note that while the egg-laying period was extended in the *eat-2* mutant, similar to the pattern exhibited by the animal carrying the *qdIs10* transgene, the total number of progeny was not significantly distinct from that of wild-type control. We observed reduced growth rate in the *eat-2* mutant, reminiscent of that in the *qdIs10* background.

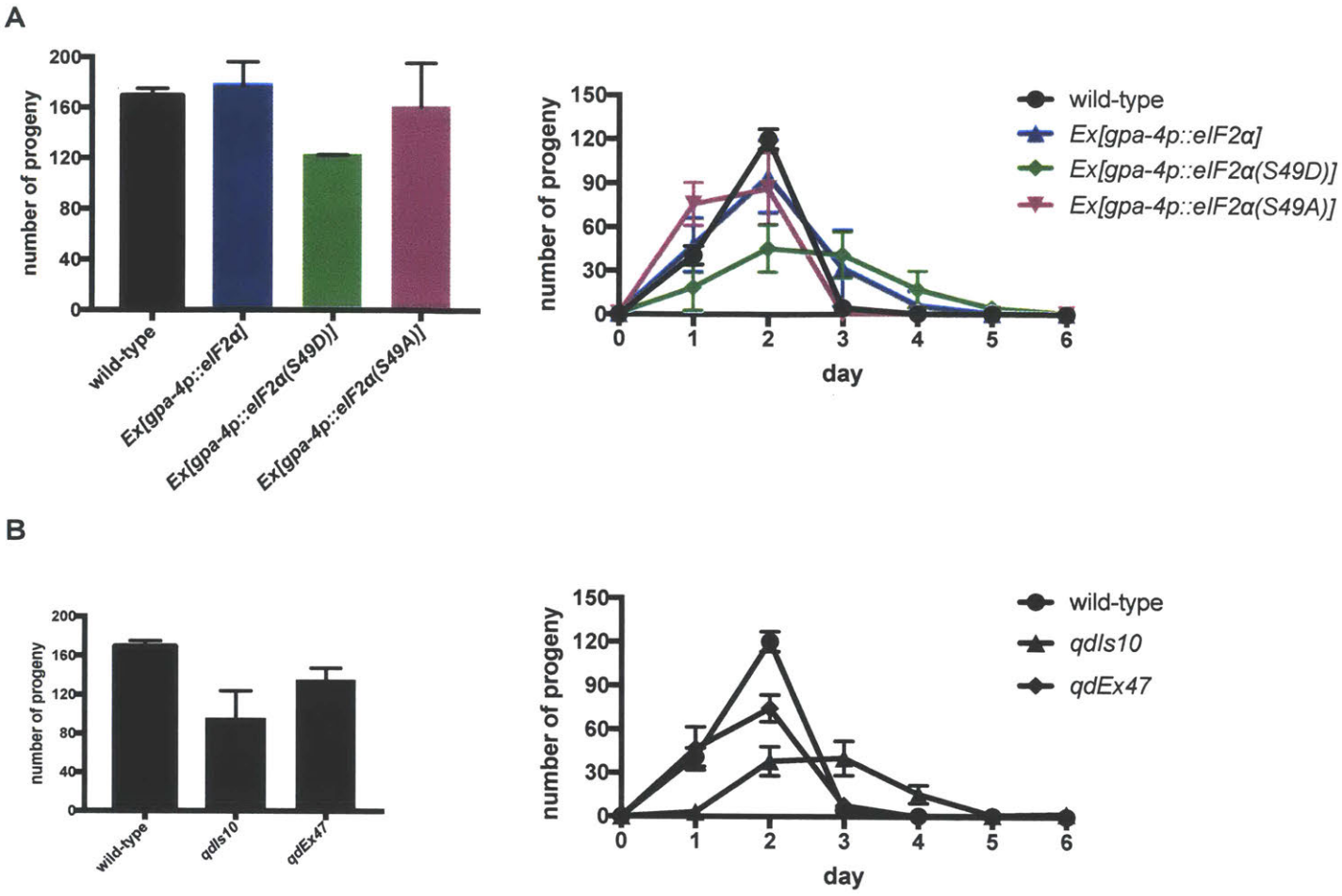


Figure S9: The starvation-like reproductive response is specific to the phosphorylation state of neuronal eIF2α and does not result from general neuronal dysfunction

A. Human caspase was expressed under the ASI-specific promoter (*daf-7p*) in the *qdEx47* transgene. Ablation of ASI was confirmed by dauer entry (Bargmann and Horvitz, 1991) and ASI-specific GFP visualization. Only animals that had entered dauer diapause and recovered were assayed for progeny production.

B. Animals harboring extrachromosomal arrays that express ASI-specific eIF2α of indicated phosphorylation status of Ser49 were assayed for total number of brood size and egg-laying duration. The animals carrying the extrachromosomal phosphomimetic neuronal eIF2α array appeared clear and small, and had reduced brood size, similar to the phenotypes conferred by the *qdIs10* transgene. We note that while the animals expressing the wild-type eIF2α appeared healthy, very few of them had somewhat clear and slightly smaller appearances, with normal progeny production. Importantly, the animals carrying the extrachromosomal unphosphorylatable neuronal eIF2α transgene exhibited wild-type developmental and reproductive phenotypes.

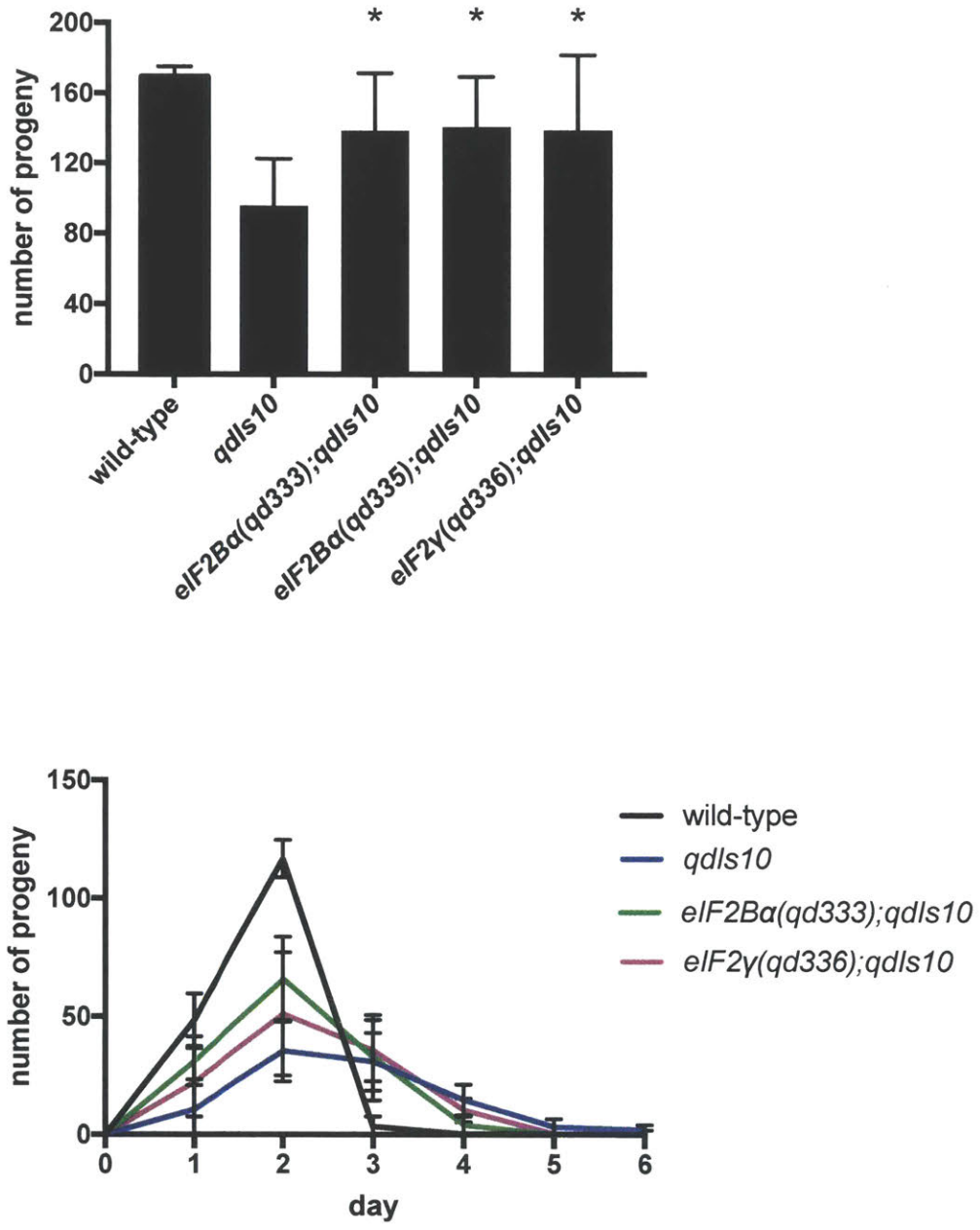


Figure S10: eIF2(αP)-insensitive mutatations significantly suppress the starvation-like organismal response induced by ectopic neuronal eIF2α phosphorylation
Animals with indicated genotypes were assayed for total number of progeny and egg-laying rate. Asterisks indicate statistical significance, $p < 0.01$, as determined by two-tailed Student's t-test (*qdIs10* versus *qdIs10* in the presence of eIF2(αP)-insensitive mutations).

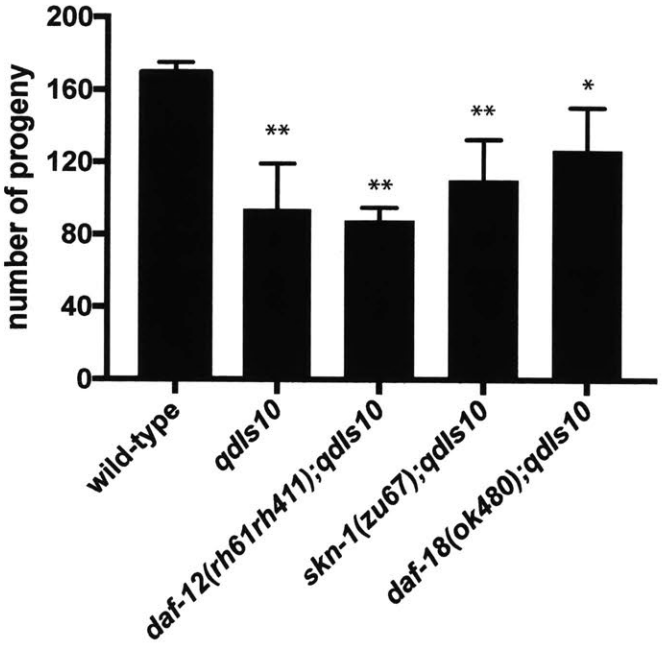


Figure S11: Examination of the roles of stress-responsive transcription factors in mediating the organismal response to neuronal eIF2α phosphorylation
Animals with indicated genotypes were assayed for progeny production. For the *skn-1* mutant background, dead eggs were scored as the mutation results in embryonic lethality. Single asterisks and double asterisks denote p values < 0.01 and around 0.01 respectively, as determined by two-tailed Student's t-test, against wild-type control.

Chapter IV

Discussions and Future Directions

In the first section of this chapter, findings that are pertinent to DAF-28/insulin biogenesis and ER physiology in the chemosensory neurons will be discussed. In the second section, observations concerning cellular and organismal sensitivity to neuronal translational control by eIF2α phosphorylation will be discussed. In the third section, I will discuss genes characterized in this study to regulate dauer formation, including class III genes. I then will conclude with perspectives on the genetic and molecular basis of sensory integration and dauer formation, particularly on cell-nonautonomous roles of stress signaling mechanisms in the worm sensory nervous system. In each section, future directions, when applicable, will be included.

Mechanistic understanding of the *daf-28(sa191)* constitutive dauer entry phenotype

The *daf-28(sa191)* Daf-c mutant was initially isolated in the Thomas group, and the molecular identity of the allele was identified in the Ruvkun group (Malone and Thomas, 1993 and Li et al., 2003). My genetic analysis in Chapter II has revealed neuronal ER stress and subsequent PERK/PEK-1 activation as a key molecular event triggering the dauer decision.

Current and previous genetic studies of the daf-28(sa191) Daf-c mutant

My findings corroborate the observations described in the Thomas and Ruvkun studies, which are as follows:

1. The dominant nature of the *daf-28(sa191)* allele was noted in a previous study, suggesting alteration of function (Malone and Thomas, 1993) that is consistent with my findings illustrating the ability of the toxic mutant DAF-28 peptide to disrupt ER homeostasis and activate the UPR in the ASI chemosensory neurons (Chapter II, Fig 1). The dominant mode of inheritance can be explained by the fact that half the dosage of the toxic gene product is sufficient to disrupt ER protein-folding homeostasis (Chapter II, Fig S1).

2. The *daf-28(sa191)* heterozygote, however, entered dauer at a low penetrance both in the Thomas study and in our hands (Malone and Thomas, 1993 and Chapter II). The relatively low dauer entry incidence observed in the *daf-28(sa191)* heterozygote may be explained by the contribution of the *daf-28* wild-type copy, which may exert anti-dauer (or pro-development) effects. In fact, if the anti-dauer influence of the wild-type *daf-28* is diminished, the pro-dauer effects of the toxic *sa191* allele are substantially potentiated, as the *daf-28(sa191)* allele in *trans* to the null alleles of *daf-28* strongly promote dauer entry (Malone and Thomas, 1993 and Chapter II, Fig 1).

The anti-dauer capacity of DAF-28 is unlikely to be a potent one, as *C. elegans* harbors forty insulin-like peptides that function redundantly in regulating the dauer decision (Pierce et al., 2001, Ritter et al., 2013 and Hung et al., 2014). Importantly, loss of both copies of *daf-28* does not result in a consistently appreciable Daf-c phenotype, as demonstrated using both previously characterized *daf-28* alleles in Chapter II (see also Ritter et al., 2013 and Hung et al., 2014) and the null allele isolated from my genetic screen in Chapter III. Notably, there are only two instances where reduced DAF-28 activity can promote the dauer decision: (i) the *daf-28* null allele in *trans* to the ER-

stress-inducing *sa191* allele strongly induces dauer formation (Chapter II, Fig 1) and (ii) loss of function of *daf-28* in combination of defects in other functionally redundant insulin genes such as *ins-4* and *ins-6* can result in appreciable constitutive dauer entry (Cornils et al., 2011 and Hung et al., 2014). These two examples indicate that *daf-28* loss of function, while not sufficient to trigger constitutive dauer entry, can enhance dauer formation in a synergistic fashion with other sensitized genetic backgrounds.

We note that the observation that overexpression of the *daf-28(sa191)* transgene only results in a weak and variable dauer arrest phenotype (Li et al., 2003) may be explained by the mild anti-dauer effect of the wild-type DAF-28, similar to the weakly penetrant phenotype exhibited by the *daf-28(sa191)* heterozygote discussed above. The dauer incidence incidence increases, albeit not at the *daf-28(sa191)* level, when the overexpression is carried out in the genetic background where the *daf-28* gene dosage was reduced by half (Li et al., 2003). We speculate that overexpression of the toxic peptide, in particular in the ASI neurons, may result in a highly penetrant Daf-c phenotype in the *daf-28* null background, reminiscent of the strong effect of constitutive expression of the phosphomimetic eIF2α on dauer diapause in the *daf-28* null background (Chapter II, Fig S3).

3. The Thomas group has shown that the *daf-28(sa191)* mutation promotes the dauer decision completely independent of the TGFβ pathway and partially dependent on the insulin pathway (Malone et al., 1996), suggesting that other signaling mechanisms may be involved. I have demonstrated that loss-of-function alleles of *pek-1*, including the eighteen new ones isolated from my genetic screen in Chapter III, are able to robustly suppress the Daf-c phenotype (Chapter II, Fig 2 and Chapter III). My identification of the

PERK/PEK-1 arm of the UPR as a key mediator of the *daf-28(sa191)* Daf-c phenotype thus complements the Thomas group's findings.

Misfolded insulin and ER stress

The hypothesis that the *daf-28(sa191)* mutation may result in a toxic gene product was initially suggested by the Ruvkun group (Li et al., 2003), and I demonstrated specifically that the neuronal UPR is activated in the presence of the mutant DAF-28 insulin peptide, indicative of sustained ER stress (Chapter II, Fig 1). My observations regarding neuronal ER stress are also confirmed in a recent independent study (Klabonski et al., 2016). Additionally, that study shows that ectopic pan-neuronal expression of the activated form of the UPR transcription factor XBP-1, which ameliorates ER stress, significantly suppresses their Daf-c phenotype (Klabonski et al., 2016). Taken together, these observations indicate that neuronal ER stress triggered by misfolded insulin is the driving molecular event underlying the constitutive dauer entry in the *daf-28(sa191)* mutant.

The ability of the R37C alteration in the mutant DAF-28 to confer toxicity in the ER is reminiscent of mutant insulins identified in the Akita diabetes mouse model, as well as in patients with permanent neonatal diabetes mellitus and mutant *INS*-gene induced diabetes of youth (Wang et al., 1999, Stoy et al., 2007 and Liu et al., 2010). These disease-associated insulin alleles carry missense point mutations, and notably the severe dominant alleles result in gain or loss of cysteine residues, which are normally present in pairs for disulfide bond formation in mature insulin peptides, including the *C. elegans* DAF-28 (Li et al., 2003). Generation of unpaired cysteine residues is

characteristic of the insulin R89C alteration identified in permanent neonatal diabetes mellitus, as well as of the insulin C96Y change that results in activation of the UPR and beta cell death in the Akita insulin-dependent diabetes mouse model (Stoy et al., 2007, Wang et al., 1999 and Oyadomari et al., 2002). Intriguingly, when all the remaining cysteine residues are mutated in the C96Y insulin mutant, the toxicity is abrogated, pointing to the critical contribution of cysteine thiol exposure to toxicity and disruption of ER homeostasis (Liu et al., 2010).

We note that the isolation of the *daf-28* revertant alleles in Chapter III further supports the findings and proposed mechanisms regarding mutant insulin toxicity. For instance, the *daf-28(sa191qd330)* Daf-c suppressor allele, which carries a missense mutation in the initiator codon, indicates that translation of the toxic R37C DAF-28 variant is critical for constitutive dauer formation. The *daf-28(sa191qd331)* results in the removal of the terminal 16-amino-acid-long section of DAF-28 that contains one cysteine residue. The elimination of that extra ninth cysteine residue restores the even number of cysteines optimal for disulfide pairing, circumventing ectopic thiol exposure resulting from the unpaired cysteine residue. To definitively confirm that toxicity to the ER is diminished or abolished in the *daf-28(sa191qd330)* and *daf-28(sa191qd331)* genetic backgrounds, the approach described in Chapter II to assess the UPR induction can be utilized in these suppressor mutants.

Roles of other ER-resident proteins in insulin toxicity

We have also identified a *C. elegans* protein disulfide isomerase family member ERp44 ortholog, C30H7.2, as a mediator of mutant DAF-28 toxicity. Functioning as an

ER-resident chaperone whose expression is induced by ER stress (Anelli et al., 2002), ERp44 is anticipated to have a role in maintaining and restoring general ER proteostasis. To our surprise, the absence of ERp44 does not confer any growth or reproduction defect phenotypes (data not shown), but instead reduces the toxicity caused by the mutant DAF-28, as indicated by diminished neuronal UPR induction (Chapter III, Fig 1C). While ERp44 is involved in the quality control of diverse ER client proteins, including proinsulin (Pottekat et al., 2013), my finding suggests that ERp44 also plays an unexpected role in participating in toxicity generated by the unpaired cysteines in the mutant DAF-28(R37C). Because ERp44 functions in thiol-mediated retention in the ER (Anelli et al., 2003), it is plausible that ERp44 forms abnormal disulfide-linked complexes with DAF-28(R37C) and assists in establishing and retaining the toxic products in the ER. Future mutational analysis to identify the ERp44 cysteine residues interacting with DAF-28(R37C) to induce the neuronal UPR will be mechanistically informative.

Based on my hypothesis that ERp44 helps retain the toxic mutant DAF-28 product, we would anticipate that *ERp44* loss of function may increase the secretion of DAF-28(R37C), thus unburdening the ER and mitigating the UPR induction. It is also conceivable that the absence of ERp44 may trigger compensatory induction of other resident chaperones that improve ER proteostasis and alleviate the toxicity triggered by the mutant insulin. Such compensatory regulation among ER chaperones, in particular the GRP78/BiP family members, has been observed in *C. elegans* (Kapulkin et al., 2005).

In light of the saturated nature of my genetic screen that yielded three null alleles of *c30h7.2/ERp44*, the absence of any recovered alleles of the genes encoding the other

two *C. elegans* ERp44 paralogs suggests that the C30H7.2 may be the only ERp44 paralog that acts on the DAF-28 insulin or in the ER of the ASI chemosensory neurons. Existing loss-of-function alleles of the other two *ERp44* paralogs can be used to test such hypothesis. While ERp44 has been studied in connection with diverse mammalian ER client proteins and tissues (Higo et al., 2005, Freyaldenhoven et al., 2012, Hisatsune et al., 2015, Yang et al., 2016), its physiological role in the worm remains unclear. My characterization of this evolutionarily conserved chaperone in the context of insulin biosynthesis and developmental plasticity thus reveals an organismal role of the chaperone in a ER-client-protein- and cell-type-specific context in *C. elegans*.

I have also identified the Daf-c suppressor mutations in genes that encode ER client proteins including DPY-3, DPY-8 and ROL-3 (Chapter III). DPY-3 and DPY-8 are cuticular collagens, synthesized prior to hatching and molting, and they both belong to a discrete interacting group of collagens (McMahon et al., 2003). Importantly, *dpy-3* and *dpy-8* are both temporally and spatially co-expressed (McMahon et al., 2003). The isolated *dpy-3* and *dpy-8* alleles both result in glycine substitutions, which occur in vertebrate collagen mutations that underlie various heritable disorders (Prockop and Kivirikko, 1995). The endogenous expression patterns of *dpy-3* or *dpy-8* can be determined, and if neuronal expression is detected, it will be intriguing to examine whether loss of function of these collagen genes results in alleviation of the UPR induction in the chemosensory neurons, underlying the suppression of the Daf-c phenotype. Notably, in the mouse Alzheimer's model, a specific type of neuronal collagen can influence proteostasis and neurotoxicity caused by aggregation of peptides (Cheng et al., 2009). *C. elegans* collagen biosynthesis requires protein disulfide

isomerases (Winter et al., 2007), and it is plausible that the absence of DPY-3 or DPY-8 may liberate the isomerases that function to improve general ER homeostasis, thus mitigating ER stress that is required for the dauer decision.

The other ER client protein ROL-3 whose absence may improve ER proteostasis to suppress the Daf-c phenotype is an ortholog of a receptor tyrosine kinase (Jones et al., 2013). Intriguingly, previously isolated hypomorphic *rol-3* alleles affect collagen processing and assembly (Jones et al., 2013). Future studies of *rol-3* in the context of dauer formation induced by neuronal ER stress should clarify its expression pattern and the ability to influence the UPR, as well as its potential interactions with collagens such as DPY-3 and DPY-8. Collectively, the recovery of *dpy-3, dpy-8* and *rol-3* alleles suppressing the dauer entry phenotype suggests that the ER toxicity elicited by misfolded insulin can be modulated by bystander client proteins.

ER stress in the sensory nervous system

The ASI chemosensory neurons that regulate dauer formation synthesize a broad array of neuropeptides to mediate multiple physiological processes (Ren et al., 1996, Schackwitz et al., 1996, Pierce et al., 2001 and Li et al., 2003). Such secretory demands may render the neuronal proteostasis readily perturbable by environmental and developmental cues that evoke neuroendocrine responses. A similar scenario has been observed in another tissue of *C. elegans*, as infection by bacterial pathogen and subsequent innate immunity activation in the intestine induce ER stress and the UPR (Richardson et al., 2010). Notably, in mammals, nutritional and metabolic fluctuations result in imbalance in ER homeostasis in multiple tissues (Hotamisligil, 2010 and Ron

and Harding, 2012). We speculate that similarly unfavorable microbial or nutritional stimuli may reprogram neuroendocrine responses in ASI, necessitating and triggering homeostatic UPR signaling in the sensory neurons that mediate diverse physiological outputs in the worm, including dauer diapause.

We note that different chemosensory neurons may have varied thresholds for ER homeostasis perturbations. In the *daf-28(sa191)* mutant, induction of the UPR is observed only in the ASI neurons, but not in the other neurons where the mutant *daf-28* is also expressed (Chapter II, Fig S1). A recent study has further confirmed spatial and temporal variations in the UPR activation caused by the DAF-28(R37C) insulin (Klabonski et al., 2016). Different expression levels of ER client proteins, including the toxic or misfolded ones, may account for the variability in susceptibility to ER stress and UPR activation among distinct cell types and life stages. Furthermore, it is plausible that some cell types are particularly sensitive to perturbations in ER proteostasis, as observed in the mammalian brain, where Purkinje cells, but not other neuronal cell types, are susceptible to misfolded proteins associated with neurodegeneration (Zhao et al., 2005 and Lee et al., 2006).

The PERK arm of the UPR and its cell-nonautonomous roles

We have established that the neuronal ER stress triggered by DAF-28(R37C) does not result in cellular death or general dysfunction (Chapter II, Fig S2). Of note, a recent study has confirmed my finding regarding normal secretion of the ASI-specific peptide in the *daf-28(sa191)* background, and additionally has engineered a DAF-28(R37C) translational fusion construct to show that even some of the mutant peptides can be

secreted (Klabonski et al., 2016). My study argues that DAF-28(R37C) promotes dauer formation through a mechanism distinct from the dauer-promoting effect of ASI death/ablation (Bargmann and Horvitz, 1991 and Chapter II, Fig S2). Specifically, the *sa191* mutation triggers dauer entry through the PERK/PEK-1 UPR signaling mechanism, as evidenced by the ability of loss-of-function *pek-1* alleles, eighteen of which were isolated from the forward genetic screen in Chapter III, to suppress the Daf-c phenotype. The absence of PEK-1 has been shown to exacerbate ER stress in other *C. elegans* tissues (Richardson et al., 2011 and Klabonski et al., 2016), and would likely further disrupt ER homeostasis in the ASI neurons where *daf-28(sa191)* is expressed. Mutations in the other arms of the UPR that similarly aggravate neuronal ER stress and thus amplify the triggering molecular event underlying dauer entry *enhance* the Daf-c phenotype (Chapter II, Fig S1). *pek-1* mutations, on the other hand, are unable to enhance dauer formation and in fact suppress the dauer entry phenotype (Chapter II, Fig 2), demonstrating that PEK-1 activation is the key signal transduction mechanism between neuronal ER health and developmental choices.

The physiological roles of PEK-1 in *C. elegans* have remained less defined than those of the other branches of the UPR, in particular the IRE-1/XBP-1 arm (Shen et al., 2001, Richardson et al., 2010, Henis-Korenblit et al., 2010, Mao and Crowder, 2010, Taylor and Dillin, 2013 and Safra et al., 2013). Of note, the functions of PEK-1 are often revealed only when the other branches of the UPR are blocked (Shen et al., 2005, Richardson et al., 2011 and Eisermann et al., 2016). The ability of *pek-1* loss of function to robustly suppress the dauer arrest phenotype therefore underscores a primary, non-redundant role of PEK-1/PERK, functioning in the chemosensory neurons to modulate

the dauer developmental decision. Of note, another physiological contribution of PERK in the sensory nervous system has been observed in the mammalian olfactory cell fate commitment (Dalton et al., 2013). In mammals, PERK has established homeostatic roles in the pancreatic insulin-producing beta cells (reviewed in Ron and Harding, 2012). Of note, the C96Y Akita insulin has been shown to induce eIF2α phosphorylation downstream of PERK activation in the pancreatic beta cell line (Hartley et al., 2010), reminiscent of the activation of the UPR by DAF-28(R37C) in the worm. Another toxic ER client peptide, the mutant murine lens gap junction protein, specifically activates PERK, resulting in ER stress and ultimately congenital cataracts (Berthoud et al., 2016). While the molecular mechanisms underlying PEK-1 activation by the toxic DAF-28 insulin remain unclear, a recent study has suggested that the luminal domain of human PERK harbors the capacity to recognize misfolded proteins (Wang et al., 2016). These observations indicate diverse roles of PERK in maintaining proteostasis in a broad array of tissues and biological contexts.

As genes that mediate dauer formation are often involved in regulation of longevity (Kenyon et al., 1993 and Larsen et al., 1995), a modest lifespan extension is noted in the *daf-28(sa191)* mutant, compared to wild-type control (Malone et al., 1996). While we did not observe a significant life extension in our hands, we speculate that, similar to the dauer entry phenotype, other organismal effects conferred by ASI-specific ER stress may be mediated to PEK-1 activation, pointing to cell-nonautonomous roles of this UPR arm in coordinating tissue-specific stress signaling with systemic responses. The molecular mechanism downstream of PEK-1 activation, which is alteration of the

cellular translational landscape by eIF2α phosphorylation, will be the subject of the next section.

Molecular determinants of cellular and systemic sensitivity to neuronal eIF2α phosphorylation

PERK is one of the four eIF2α kinases, constituting the Integrated Stress Response that attenuates protein synthesis under adverse conditions in mammals (reviewed in Sonenberg and Hinnebusch, 2009). In *C. elegans*, only PEK-1/PERK and GCN-2 are the kinases activated by disruption of ER homeostasis and amino acid deprivation, respectively. I have shown that phosphorylation of Ser49 of *C. elegans* eIF2α in the ASI neurons promotes the dauer developmental decision (Chapter II). The robust Daf-c phenotype conferred by neuronal eIF2α phosphorylation is amenable to the forward genetic approach described in Chapter III, allowing me to recover genes governing responsiveness to phosphorylated eIF2α (Class II genes). Class II genes are specific to cellular and systemic sensitivity to eIF2(αP), and are not involved in misfolded insulin biosynthesis or general dauer neuroendocrine responses. Class II genes *eIF2Bα* and *eIF2γ* have not previously been genetically characterized in *C. elegans*, particularly in the context of organismal development.

Genetic analysis of eIF2Bα in C. elegans

eIF2α phosphorylation attenuates protein synthesis by binding and inhibiting the eIF2B guanine nucleotide exchange factor that activates eIF2 recycling required for initiation of translation. The regulatory subunit eIF2Bα is critical for recognizing

eIF2(αP), ultimately resulting in suppression of the eIF2B exchange activity. The genetic requirement of eIF2Bα for mediating dauer entry triggered by ASI-specific phosphorylation of eIF2α reveals a novel organismal role of eIF2Bα, in addition to its cellular function previously characterized in yeast (Yang and Hinnebusch, 1996).

While there are three regulatory subunits of eIF2B that are susceptible to inhibition by phosphorylated eIF2α, we isolated eleven loss-of-function alleles of the gene encoding the α subunit of eIF2B, but no alleles of the genes encoding the other two regulatory subunits, β and δ, were recovered. Null alleles of *eIF2Bβ* and *eIF2Bδ* were not anticipated because these two subunits are essential, due to molecular functions critical to translation initiation that are unrelated to their dispensable regulatory roles in recognizing eIF2(αP) (Dev et al., 2010). Notably, a respectable number of *S. cerevisiae* hypomorphic alleles of *GCD7/eIF2Bβ* and *GCD2/eIF2Bδ* that render the yeast cells refractory to the inhibitory effects of eIF2α phosphorylation have been characterized (Vazquez de Aldana and Hinnebusch, 1994). It is plausible that, unlike in yeast, partial loss of function of the β and δ subunits may be less tolerable in *C. elegans*. However, in light of the saturated nature of the screen that yielded eleven alleles of the α subunit gene suppressing the Daf-c phenotype, we also surmise that the complete lack of partial loss-of-function alleles of the β and δ subunit genes recovered may be due to the prominent roles of the α subunit, compared to the other regulatory eIF2B subunits, in conferring sensitivity to eIF2(αP) in particular neuronal cell types or tissues. Examination of relative abundance of eIF2B subunits in relevant cell types may illuminate tissue-specific roles of distinct regulatory subunits mediating eIF2(αP) sensitivity. The functional and structural heterogeneity among the eIF2B regulatory subunits is also suggested by the finding that the mammalian

eIF2Bδ, not α or β, is the molecular target of the drug ISRIB that induces eIF2(αP) insensitivity (Sidrauski et al., 2015 and Sekine at al., 2015). We note that, similar to most yeast eIF2(αP)-insensitive alleles of *eIF2Bα, eIF2Bβ* and *eIF2Bδ*, all *C. elegans eIF2Bα* alleles in my study do not impair growth and reproduction under normal growth conditions, indicating the conserved functions of eIF2B regulatory subunits that are dispensable for basal translation initiation, but are required for stress-induced inhibition of protein synthesis mediated by eIF2(αP).

We note that one of our isolated *eif-2B.1/eIF2Bα* alleles affecting the highly conserved residue, threonine 41, behave similarly to the well-characterized T41A alteration in the *S. cerevisiae* eIF2Bα ortholog GCN3, which contributes to resistance to eIF2α phosphorylation in response to nutrient deprivation in yeast (Chapter III, Hannig and Hinnebusch, 1988 and Pavitt et al., 1997). Moreover, the T41A alteration in human eIF2Bα enhances susceptibility to viral infection due to the inability to inhibit translation in response to viral RNA-induced eIF2α phosphorylation (Elsby et al., 2011). The observed insensitivities to phosphorylated eIF2α in multiple species suggest a critical role of the T41 residue, as well as of the alpha subunit, in translational control mediated by eIF2α phosphorylation. Similar to the T41I substitution in EIF-2B.1, the majority of the recovered alleles alter the conserved residues of eIF2Bα, suggesting critical contributions of these residues to recognizing phosphorylated eIF2α to exert translational control (Chapter III, Fig S3 and Kuhle et al., 2015). Our identification of diverse residues essential for eIF2(αP)-sensitivity may provide resources for further structure-function studies of eIF2B regulation in *C. elegans*, and potentially higher metazoans, as in the case of the T41A eIF2(αP)-resistant mutants in yeast and human.

All eleven *eif-2B.1/eIF2Bα* alleles isolated confer strong suppression of the Daf-c phenotype. One of the alleles, *qd335*, gives rise to in an alternative translation start site in the 5′ UTR, a few base pairs upstream of the original ATG, resulting in a severely truncated peptide, thus considered presumptive null. A previously isolated deletion allele, *pk720*, is also used in this study to confirm the suppression. The isolation of multiple alleles of the dispensable *eIF2Bα* that behave like nulls is consistent with their loss-of-function nature, in contrast to recovery of single alteration-of-function alleles of the other essential genes in Class II, *eIF2Bα* and *eIF2γ*. While both loss-of-function *eIF2Bα* alleles strongly suppress the dauer entry phenotype, indicating complete insensitivity to the effects of neuronal eIF2(αP), the alternate start site allele confers a recessive suppression phenotype, while the deletion allele a dominant one. It is plausible that the deletion allele heterozygosity results in reduction of *eIF2Bα* dosage by half, whereas the alternate start site heterozygote may be more leaky in expressing *eIF2Bα*, thus slightly increasing the *eIF2Bα* dosage to the level that permits some sensitivity to neuronal eIF2(αP) and its dauer-promoting effects. Eight out of ten of the isolated *eIF2Bα* missense mutations confer a dominant suppression phenotype similar to the deletion allele, indicating that the eight residues affected may be critical for eIF2Bα functional and structural integrity, and that their disruptions severely impair the α subunit function in eIF2(αP) responsiveness, even in the heterozygous configuration (Chapter III, Fig S4). Indeed, four of the eight residues are not only conserved among eIF2Bα across species, but also among the equivalent domains in the other regulatory subunits of eIF2B that recognize eIF2(αP), β and δ, suggesting pivotal regulatory functions. Only two out of ten of the isolated *eIF2Bα* missense mutations confer a recessive Daf-c suppression phenotype and affect residues

that are not evolutionarily conserved (Chapter III, Fig S4). We speculate that while reduction of function caused by these two recessive alleles in the homozygotes may be sufficient to abolish eIF2(αP) sensitivity similar to the nulls, in the heterozygotes, the dosage or functional residuals, if any, of the mutant proteins may not be sufficient to overcome the ability of the wild-type counterparts to confer susceptibility to eIF2(αP). Taken together, these ten residues are essential for eIF2Bα to function to mediate the inhibitory effects on eIF2B, translation and dauer development, although further biochemical analyses will be required to demonstrate that contributions of each residue to eIF2Bα function, structure and expression may be heterogeneous.

Genetic analysis of eIF2γ in C. elegans

The G-protein eIF2 subunits are essential for protein synthesis integral to *C. elegans* development and reproduction. We isolated rare alteration-of-function alleles of genes encoding eIF2 subunits, *eIF2α* and *eIF2γ* (Chapter III). The alteration in the Ser49 phosphoryaltion site of eIF2α(S49F) conferred by the *eif-2.1/eIF2α(qd338)* Daf-c suppressor allele corroborates our model that efficient eIF2α phosphorylation in the ASI neurons is required for the dauer developmental decision. This allele also supports our observation that overexpression of another unphosphorylatable version of eIF2α(S49A) in the two neurons only partially suppresses the *daf-28(sa191)* dauer entry phenotype (Chapter II, Fig 3). The partial Daf-c suppression mediated by the transgene could be accounted for by the contribution of the endogenous, readily phosphorylatable eIF2α (Chapter II, Fig 3), whereas in the *eif-2.1/eIF2α(qd338)* mutant expressing only

eIF2α(S49F), eIF2α phosphorylation is abrogated in all tissues and the Daf-c suppression is hence complete (Chapter III, Fig 2).

I also identified a substitution in the conserved Ser443 of eIF2γ as a product of the *eif-2.3/eIF2γ(qd336)* Daf-c suppressor allele. We hypothesized that the eIF2γ(S443L) alteration abolishes sensitivity to phosphorylation of eIF2α by rendering an essential catalytic subunit of eIF2B dispensable, therefore bypassing a subset of functions of eIF2B that may otherwise be subject to the inhibitory effects of eIF2(αP) (Chapter III, Fig 3). I then showed that the eIF2γ(S443L) change permits the animals to develop normally in spite of the growth-inhibiting condition where the essential eIF2B subunit γ (PPP-1 in *C. elegans*) is knocked down by RNAi (Chapter III, Fig 3). We predict that *eIF2γ(S443L)* expressed in ASI also bypasses the need for the PPP-1 eIF2B subunit, liberating the neurons from the dauer-promoting effects of eIF2(αP), which acts on and sequesters eIF2B. The other class II alleles of *eIF2α* and *eIF2Bα* do not result in obviating the developmental requirement of the PPP-1 eIF2B subunit (Chapter III, Fig S5), indicating distinct mechanisms that confer eIF2(αP) insensitivity among mutations in these three translational regulatory factor genes:

1. Removal of the Ser49 phosphorylation site in the *eif-2.1/eIF2α(qd338)* mutant
2. Impairment of the subunit of eIF2B that recognizes eIF2(αP) and transduces its pro-dauer effects in the *eif-2B.1/eIF2Bα* mutants
3. Bypassing the requirement of an essential catalytic subunit of eIF2B, diminishing the cellular reliance on eIF2B and its sequestration by eIF2(αP) in the *eif-2.3/eIF2γ(qd336)* mutant

We note that the *eIF2γ(S443L)* mutation does not result in general translation attenuation, as demonstrated by normal growth and reproduction, but specifically alters the sensitivity to the dauer-promoting consequences of phosphorylation of eIF2α. As summarized in Chapter III, there are two categories of previously isolated eIF2 subunit alleles in yeast:

1. eIF2 subunit mutations that affect general translation initiation steps such as those altering start codon recognition (Donahue et al., 1988, Alone et al., 2008 and Cigan et al., 1989) or impairing global protein synthesis and cellular growth (Erickson and Hannig, 1996 and Borck et al., 2012).
2. eIF2 subunit mutations that specifically abolishes eIF2(αP) sensitivity without impairing global translation. Examples include mutations in the yeast *eIF2α* that weaken interactions required for the inhibitory effects of eIF2(αP), including the interactions between eIF2α and eIF2α kinases or between eIF2(αP) and its target eIF2B (Vazquez de Aldana et al., 1993 and Dey et al., 2005). A recent study has also characterized an *eIF2β* mutant that does not diminish general proteins but alters eIF2(αP) sensitivity (Jennings et al., 2016).

The *eIF2γ(S443L)* mutation belongs to the second category of eIF2 subunit alterations, as only the eIF2(αP)-mediated translational control, not basal protein synthesis, is affected. The *C. elegans eIF2γ(S443L)* mutation evades the inhibitory effects of eIF2(αP) via a distinct mechanism from the aforementioned yeast *eIF2β* mutant (Jennings et al., 2016). Specifically, the yeast *eIF2β* mutant bypasses the requirement for eIF2Bε—not the γ/PPP-1 subunit—diminishing the reliance on eIF2B, liberating

translation from regulation by eIF2(αP)-mediated eIF2B sequestration (Jennings et al., 2016).

The *C. elegans eIF2γ(S443L)* alteration in fact represents a novel class of *eIF2γ* mutations distinguishable from those previously characterized that only impair gross translation and cell growth (Erickson and Hannig, 1996, Alone et al., 2008 and Borck et al., 2012). One notable exception is the yeast *GCD11/eIF2γ(K250R)* mutant that not only manifests growth defects but also circumvents translational control by eIF2α phosphorylation (Erickson et al., 2001). The yeast *GCD11/eIF2γ(K250R)* mutation, however, mediates sensitivity to eIF2(αP) through a distinct mechanism from the *C. elegans eIF2γ(S443L)* mutation. Whereas the *eIF2γ(S443L)* mutant bypasses the functional requirement only for the PPP-1/eIF2Bγ subunit in the worm (Chapter III, Fig 3), the yeast *GCD11/eIF2γ(K250R)* mutation is able to functionally substitute for the absence of eIF2α and *all* eIF2B subunits. The yeast K250R alteration in eIF2γ has been postulated to augment spontaneous guanine nucleotide dissociation, rendering eIF2Bε that catalyzes the nucleotide exchange dispensable and therefore abrogating the cellular reliance on eIF2B and its sequestration by eIF2(αP). Such mechanism cannot explain eIF2(αP) insensitivity conferred by the worm *eIF2γ(S443L)* mutation because eIF2Bε is still required. Because eIF2γ(S443L) can functionally substitute for the PPP-1 subunit, but not for eIF2Bε, the alternative mechanism is likely to involve a molecular event catalyzed exclusively by PPP-1, which is not the GTP-GDP exchange that is largely mediated by eIF2Bε.

The PPP-1 subunit has been shown to participate in the dissociation of the translational regulatory factor eIF5 that binds to eIF2-GDP and antagonizes eIF2B

(Jennings et al., 2013). It is plausible that the S443L change in eIF2γ facilitates the displacement of eIF5, bypassing the requirement for PPP-1 in dissociating eIF5 from eIF2 to form a translation-competent eIF2 complex. The augmented dissociation of eIF5 contributed by eIF2γ(S443L) likely potentiates the eIF2B function, counteracting the inhibitory effects of eIF2(αP) on eIF2B. Furthermore, in addition to sequestering eIF2B, phosphorylation of eIF2α has been postulated to enhance the abundance of the eIF2-eIF5 complex, restricting productive recycling of eIF2 (Jennings and Pavitt, 2010). Therefore, the eIF2γ(S443L) alteration that destabilizes and depletes the eIF2(αP)-induced translation-incompetent eIF2-eIF5 complex would undermine such inhibitory consequences of eIF2α phosphorylation.

Sensitivity to eIF2α phosphorylation and animal physiology

In addition to the organismal roles of the translational regulatory factors eIF2Bα and eIF2γ in regulating the dauer decision, we also confirmed their cellular functions in mediating sensitivity to eIF2(αP). Similar to the eIF2(αP)-mediated stress-induced transcripts in mammals (Harding et al., 2000 and Vattem and Wek, 2004), the *C. elegans ATF-5* mRNA bearing an upstream open reading frame (uORF) is preferentially translated under conditions where eIF2α is phosphorylated. Therefore, a fluorescent reporter carrying the *ATF-5* uORF is utilized as a readout for the cellular responses to eIF2(αP) (provided by David Ron's group). As noted in Chapter III, under basal conditions, the reporter is induced substantially in the spermatheca, which may reflect the imbalance in ER homeostasis that activates the eIF2α kinase PEK-1, as the other branch of the UPR is also upregulated in this tissue (Calfon et al., 2002). We mainly utilize this

system to illustrate that all mutations in the Class II genes (*eif-2.1/eIF2α, eif-2B.1/eIF2Bα* and *eif-2.3/eIF2γ*), which contribute to organismal insensitivities to neuronal eIF2(αP), also abolish cellular responses to physiological eIF2α phosphorylation occurring in the spermatheca (Chapter III, Figs 2B and 3C). These observations indicate that eIF2Bα and eIF2γ function in both the sensory neurons and the spermathecal cells to mediate sensitivity to eIF2α phosphorylation. The biological consequences of induced spermathecal eIF2α phosphorylation are beyond the scope of my study, but I surmise that calcium-mediated contraction of the structure required for ovulation may perturb ER homeostasis, necessitating the UPR (Bui and Sternberg, 2002). Defects in cellular responses to the UPR and eIF2α phosphorylation might result in aberrant oocyte movement from the gonad or to the uterus, affecting fertility.

We asked if the inability to properly respond to eIF2α phosphorylation affects other physiological phenotypes beyond the constitutive dauer entry triggered by neuronal ER stress. The roles of eIF2α phosphorylation in dauer formation in response to other stimuli will be discussed in the final section of this chapter. Only non-dauer physiology will be the focus of this section. We found that eIF2α phosphorylation is not required for normal larval development upon exposure to conditions that are associated with disruption of ER homeostasis that may induce phosphorylation of eIF2α through PEK-1 such as pathogenic bacteria, heat and the ER-toxic drug tunicamycin (Chapter III, Fig S7). The absence of any developmental defects induced by the stressors suggests that eIF2α phosphorylation downstream of ER stress perception may function redundantly with other compensatory, protective homeostatic mechanisms, including the other arms of the UPR. Alternatively, since I have only focused on larval development, the life

stages where ER stressor-mediated effects are observed (Richardson et al., 2011 and Cattie et al., 2016), it is plausible that the physiological contributions of or requirements for translational control mediated eIF2α phosphorylation might be modulated by reproductive maturation and ageing. We note that we may also have not identified the specific stressor conditions that necessitate the non-redundant roles of eIF2(αP) responsiveness.

We note that while PEK-1 is required for larval development at 25°C only when the major arm of the UPR IRE-1/XBP-1 is blocked, eIF2(αP) sensitivity mediated by eIF2Bα, which presumably functions in the tissues affected by the IRE-1/XBP-1 absence, is dispensable in that context (Chapter III), raising a possibility of eIF2(αP)-independent functions of PEK-1. The possibly versatile roles of eIF2α kinases beyond phosphorylating their target eIF2α are tantalizing but beyond the scope of this study.

Because eIF2α phosphorylation is an evolutionarily conserved translational control mechanism, the dispensability of eIF2(αP) responsiveness for survival under specific sets of adverse laboratory conditions is somewhat surprising. As mentioned above, it is conceivable that ecologically relevant stressors that exclusively require eIF2(αP) signaling may remain to be identified. In contrast, the prominent roles of eIF2α phosphorylation and its associated molecular determinants (encoded by the Class II genes) in regulation of the dauer decision by sensory neurons underscores neuronal functions of this ancient translational control pathway. Such predominant contributions of eIF2α status modifications in the sensory nervous system is reminiscent of vital roles of eIF2α phosphorylation in the mammalian neurons to control complex behaviors discussed in the next sub-section.

Neuronal eIF2α phosphorylation status and animal physiology

While cellular consequences of eIF2α phosphorylation have been extensively characterized in yeast (reviewed in Sonenberg and Hinnebusch, 2009), physiological systemic roles of the conserved translational control mechanism have been highlighted in recent studies, particularly in the nervous system. Specifically, a broad array of complex biological processes including foraging, learning, addiction and imprinting have been associated with responses to eIF2α phosphorylation (Hao et al., 2005, Costa-Mattioli et al., 2007, Stern et al., 2013, Huang et al., 2016 and Batista et al., 2016). In this study, I have shown that aberrant translational status in the sensory nervous system can modulate *C. elegans* larval developmental plasticity and the starvation response during adulthood. The effects of neuronal eIF2α phosphorylation on the dauer developmental decision will be discussed in the final section of this chapter, in the context of integration of adverse sensory cues. This section will focus on the effects of constitutive eIF2α phosphorylation in the adult animals, mimicking the starvation response.

We demonstrated that constitutive phosphorylation of eIF2α in the ASI chemosensory neurons results in defects in growth rate, progeny production and fat storage (Chapter III, Fig 5). These systemic impairments are similar to those manifested when the animals are exposed to nutritionally limited conditions. That the organismal effects of neuronal eIF2(αP) on the adult worms mimic a starvation response is consistent with the triggering molecular event (eIF2α phosphorylation) and its site of action (ASI neurons):

1. eIF2α phosphorylation is an evolutionarily conserved mechanism of nutrient detection in the unicellular yeast, as uncharged tRNAs activate the GCN2 eIF2α kinase to attenuate translation while selectively inducing expression of genes that facilitate amino acid biosynthesis (Hinnebusch, 1994 and Wek et al., 1995). Importantly, in metazoans, the interplay between neuronal eIF2(αP)-mediated nutritional regulation and organism-wide responses has been demonstrated, for instance, in the GCN2-dependent rejection of amino-acid-deficient food sources in mammals (Hao et al., 2005 and Maurin et al., 2005).
2. The ASI chemosensory neurons play critical roles in food perception to mediate a diverse array of physiological outputs in non-dauer animals including lifespan extension, germline proliferation and satiety quiescence (Bishop and Guarente, 2007, Entchev et al., 2015, Dalfo et al., 2012 and Gallagher et al., 2013). It is plausible that food perception involves detection of both nutrients as well as chemosensory cues associated with growth-promoting microbial milieu.

Genetic and mechanistic basis of the cell-nonautonomous effects of neuronal eIF2(αP)

For simplicity, the developmental and reproductive defects in the adult animals discussed above, induced by the ASI-specific phosphomimetic *eIF2α(S49D) qdIs10* transgene, will be termed "starvation response." Genetic ablation of the ASI neurons does not result in the starvation response (Chapter III, Fig S9), indicating that constitutive phosphorylation of eIF2α promotes the developmental and metabolic defects through

mechanisms that do not involve general neuronal dysfunction or cell death. We speculate that the molecular consequences of phosphorylation of eIF2α the ASI neurons is remodeling the proteome landscape, in particular the neuromodulator class, resulting in a net starvation signaling output influencing the organism as a whole. Such outcomes of eIF2α phosphorylation on ASI could be mediated through (i) global, non-lethal translation inhibition and/or (ii) preferential upregulation of uORF-bearing, stress-responsive transcripts. If general translation attenuation is a predominant event underlying the starvation response, we would anticipate that other independent mechanisms diminishing translation such as inhibition of ribosomal proteins, translation regulatory factors or the TOR pathway, specifically in ASI, should confer similar effects on the worm.

In light of the abundance of examples in the literature demonstrating various organismal roles of ASI through neuropeptide modulation, it is plausible, but perhaps less likely, that eIF2(αP)-mediated translation attenuation and/or upregulation of stress-induced transcripts may influence the neuronal electrophysiological properties, as well as synaptic strengths and targets, to coordinate systemic starvation responses.

If there are non-redundant hormonal signals mediating the effects of neuronal eIF2α phosphorylation, systematic loss-of-function analyses of genes encoding neuropeptides secreted from the ASI neurons may be informative. Complete ASI-specific transcriptome and translatome should first be elucidated, under both basal and eIF2(αP)-induced conditions. The functional relevance of ASI-specific uORF-bearing transcripts that are affected at the translational level by eIF2(αP) can be genetically assessed. Forward genetic methods can be utilized as an unbiased, complementary strategy.

However, it is likely that the neuronal-eIF2(αP)-mediated starvation response results from overlapping contributions from multiple modulators, hence limiting the power of such approaches. Thus far, I have determined that reduction in insulin or TGFβ signaling, whose ligands are produced in ASI, is unable to suppress the organismal effects of neuronal eIF2(αP) in the adult animals (data not shown). A genetic suppressor screen has not been performed based on the adult starvation response phenotype induced by the *qdIs10* transgene.

In comparison, the *daf-28(sa191)* Daf-c suppresor screen in Chapter III interrogates the organismal effects of neuronal eIF2(αP) at a different life stage (pre-dauer larval development) and in the absence of wild-type DAF-28, which may subtly modulate insulin signaling. Moreover, we note that the *daf-28(sa191)* mutant is phenotypically distinct from the animal carrying the *qdIs10* transgene in two key regards. First, while the *daf-28(sa191)* mutant is Daf-c, expression of the *qdIs10* transgene does not induce dauer formation under the same condition where food is abundant and population density is low. Second, while ectopic expression of ASI-specific phosphomimetic eIF2α results in the growth and reproductive defects, the *daf-28(sa191)* animals appear wild-type and fecund throughout adulthood. The phenotypic distinctions between the *daf-28(sa191)* mutant and the *qdIs10* transgenic animal may be accounted for by the following factors:

1. The starvation phenotype is dependent upon and sensitive to the expression levels of the phosphomimetic eIF2α multi-copy transgene. For example, in the *qdIs10* heterozygote where the transgene dosage is reduced by half, the starvation phenotype is not observed, while the co-injection transformation

marker is still expressed. RNAi knockdown that non-specifically affects transgene expression can partially silence *qdIs10* to the level sufficient to blunt its ability to promote the starvation-like effects. Therefore, it is plausible that the *daf-28(sa191)* mutant does not generate enough ER toxicity in ASI to induce eIF2α phosphorylation to the same extent as the *qdIs10* homozygote.

2. While the larval neurons are susceptible to the ER-toxic and eIF2(αP)-mediated effects of DAF-28(R37C), the *daf-28(sa191)* adult neurons may have improved ER homeostasis or overall reduction in PEK-1 activation, resulting in diminished neuronal eIF2α phosphorylation, compared to the *qdIs10* multi-copy transgene. A direct approach to measure the phosphorylation level of eIF2α can be used to address whether eIF2(αP) is detected substantially and consistently throughout life in the *daf-28(sa191)* background, as my current method to assess neuronal ER stress relies on the non-PEK-1 branch of the UPR.
3. While thus far genetic manipulation of various key stress-responsive genes (Chapter III, Fig S11) has not been able to suppress the neuronal-eIF2(αP)-induced starvation response phenotype, a few non-genetic conditions have been found to diminish the starvation response. For instance, when the non-dauer *qdIs10* adults are exposed to the dauer-promoting pheromone or plates without peptone, they appear more similar to wild-type control, suggesting that chemosensory and nutritional experiences, likely to be perceived by the ASI neurons, can modulate the effects induced by constitutive eIF2α phosphorylation. Therefore, it is plausible that, even if DAF-28(R37C) could

induce sufficiently high level of eIF2α phosphorylation during adulthood, the life history (for example, having exited dauer) and metabolic context (for example, the absence of DAF-28) may mimic chemosensory or nutritional experiences that modulate the capacity of the stressed ASI to trigger the starvation response.

4. The differential effects of constitutive eIF2α phosphorylation on dauer formation in the *daf-28(sa191)* and *qdIs10* backgrounds will be discussed in the final section of this chapter.

Notwithstanding the phenotypic differences between the *daf-28(sa191)* allele and the *qdIs10* transgene, Class II genes which encode the molecular determinants of organismal sensitivity to eIF2(αP) play critical roles in mediating both the *daf-28(sa191)* Daf-c phenotype and the *qdIs10* starvation response (Chapter III). Specifically, eIF2(αP)-insensitive mutations suppress the organismal, cell-nonautonomous effects of ASI-specific eIF2α phosphorylation in both the *daf-28(sa191)* and *qdIs10* genetic circumstances. These observations illustrate common molecular factors and events downstream of neuronal eIF2α phosphorylation governing developmental plasticity and nutritional stress response throughout life.

Perspectives on genes mediating the dauer developmental decision

In this section, I will reiterate the classification of genes identified from the screen in Chapter III, before describing and discussing Class III genes.

Classification of alleles isolated from the daf-28(sa191) Daf-c suppressor scren

Now that both the *daf-28(sa191)* Daf-c phenotype and the *qdIs10* starvation response phenotype have been clarified in the previous section, I will summarize how the categorization is performed based on these two phenotypes:

Class I group: Genes in this class only mediate misfolded insulin biosynthesis or ER homeostasis. Therefore, alleles from this class, should have no effect on the *qdIs10* starvation response as neither toxic ER client proteins nor UPR activation is involved in or downstream of expression of the phosphomimetic *eIF2α* transgene. Alleles from this class should also have no effect on constitutive dauer entry induced by mutations in other dauer-regulating pathways that do not perturb ER homeostasis. For instance, *pek-1* loss of function has no effect on dauer formation induced by insulin receptor or TGFβ receptor single mutants (Chapter II, Fig 2). Class I genes should act in the ASI neurons to modulate ER proteostasis, and their functional relevance in the neurons can be assessed by measuring UPR induction in the presence or absence of the genes.

Class II group: Genes in this class encode molecular determinants of sensitivity to eIF2α phosphorylation. Hence, alleles from this class should suppress both the *daf-28(sa191)* constitutive dauer entry and the *qdIs10* starvation response, as both phenotypes are consequences of neuron-specific phosphorylation of eIF2α (Chapter III). Alleles from this class should have no effect on other dauer signaling pathways that do not require sensitivity to eIF2(αP). For example, *eif-2B.1/eIF2Bα* loss of function does not affect the Daf-c phenotype caused by diminished insulin signaling (Chapter III). Class II genes should function in the sensory neurons to modulate dauer entry, as well as in other tissues to mediate other biological processes under conditions where eIF2α phosphorylation is induced by environmental or developmental stressors.

Class III group: Genes in this class encode signal transduction molecules that function in the dauer decision-making tissues, such as the sensory nervous system, or in the target tissues that need to be reprogrammed for the long-lived, stress-resistant diapause stage. Alleles from this class should have no effect on the *qdIs10* starvation response phenotype, unless the genes also have a functionally and temporally distinct function in communicating nutritional signals to other tissues during adulthood. Thus far, I have not identified Class III genes that could suppress both the Daf-c and starvation response phenotypes. Alleles from this class should also not modulate DAF-28 biosynthesis or ER homeostasis in the ASI neurons.

Class III genes and insulin signaling

Because the *daf-28(sa191)* Daf-c phenotype in part relies on diminished insulin signaling, as evidenced by the suppression by the absence of DAF-16/FOXO downstream of the insulin receptor (Malone et al., 1996), it is plausible that some Class III genes may function as part of or to modify the insulin signaling mechanism.

To my surprise, no alleles of *daf-16* in this presumptively saturated screen have been isolated, which could indicate that DAF-16 loss of function may confer pleiotropies that obfuscate simple identification of healthy, non-dauer animals, as was the case with other suppressors. Of note, a previous study has shown that while *daf-16* mutations partially diminish the dauer formation incidence (to around 50%; significantly lower our hands, likely due to different genetic backgrounds or population densities), all non-adult *daf-16;daf-28(sa191)* animals are in fact partial dauers (Malone et al., 1996). Partial dauers have previously been described elsewhere (Vowels and Thomas, 1992).

Therefore, DAF-16 seems to be involved in two separate molecular events underlying the Daf-c phenotype: (i) the dauer decision-making process, as DAF-16 loss of function substantially reduces the incidence of diapause and (ii) the dauer remodeling process, as DAF-16 is also required for complete morphological and metabolic transformations integral to *bona fide* dauer formation. We note that none of the Class III genes that modulate insulin signaling harbor both functions of DAF-16. In fact, all the Class III genes only mediate the decision-making step (i), and no partial dauers (symptomatic of defects in function (ii)) are observed in any non-*daf-16* genetic backgrounds, suggesting that DAF-16 harbor a unique function in dauer tissue remodeling. The respectable 50% partial dauer incidence of the *daf-16;daf-28(sa191)* mutant implies that additional DAF-16/insulin-independent mechanisms, such as neuronal ER stress and eIF2α phosphorylation, operate in the *daf-16;daf-28(sa191)* mutant to promote the decision to form (partial) dauers.

Because the phenotypic criterion for the screen has been stringent (only young adults were isolated; dauers or partial dauers were not recovered, even though they could carry incompletely penetrant suppressor alleles), the partial Daf-c suppression by *daf-16* loss of function may account for the lowered probability of recovering new *daf-16* alleles, as well as alleles of other genes that may share similar functions and attributes.

Class III genes that likely function to modulate insulin signaling are as follows:

1. Five alleles of *pkc-1* were isolated (Table 1). All the alleles confer a recessive Daf-c suppression phenotype, consistent with their loss-of-function nature. *pkc-1* encodes an ortholog of mammalian protein kinase C, specifically the novel subgroup (nPKC), which is activated by diacyglycerol. In *C. elegans*,

pkc-1 is expressed in chemosensory neurons and is involved in neuropeptide secretion in motor neurons (Land et al., 1994 and Sieburth et al., 2007). Importantly, *pkc-1* is required for the constitutive dauer entry phenotype in the insulin signaling-defective mutants (Monje et al., 2011). PKC-1 has been shown to function in subsets of sensory neurons to influence the dauer decision (Monje et al., 2011).

It is striking that five alleles of *pkc-1* were recovered from our screen (only one allele was isolated from the insulin/PI3K suppressor screen of 100,000 haploid genomes in Monje et al., 2011), suggesting that the synergistic interactions between neuronal eIF2α phosphorylation and insulin signaling in promoting dauer formation in the *daf-28(sa191)* mutant may significantly depend on PKC-1 activity.

2. One allele of *daf-2* encoding the insulin receptor was isolated (Table 1). The Daf-c suppression phenotype conferred by this allele is dominant, suggesting gain or alteration of function. The Glu1240 altered is an evolutionarily conserved residue residing in the tyrosine kinase catalytic domain. I hypothesize that the alteration may result in constitutive DAF-2 receptor activation, inducing cytoplasmic sequestration of DAF-16, thus inhibiting the DAF-16-dependent dauer decision.
3. We isolated three alleles of distinct genes that likely function in neuronal/sensory signal transduction pathways. Epistasis analyses suggest that all of them interact with insulin signaling. The allele names are *qd318*, *qd369* and *qd370*.

We also isolated three alleles of *daf-12*, which encodes the steroid hormone receptor functioning downstream of all neuroendocrine signaling pathways regulating dauer formation.

Perspectives on the daf-28(sa191) Daf-c suppressor screen

Class I and Class II gene products represent novel cell-nonautonomous modulators of the dauer developmental decision, and also have independent cell-autonomous functions in previously established homeostatic processes, proteostasis and translational control, respectively. Most of the Class III gene products function to modulate the insulin signaling pathway. Despite the exhaustive nature of the screen, I have not identified Class III genes that function specifically to mediate the systemic effects of neuronal eIF2α phosphorylation; those genes would mediate both the dauer entry and starvation response evoked by neuronal eIF2(αP). I had anticipated that the forward genetic approach may reveal relevant genes whose mRNAs carry uORFs or encode neuromodulators, functioning downstream of neuronal eIF2α phosphorylation. Functional redundancies among such stress-responsive signaling molecules involved may limit the utility of single-gene-based loss-of-function analyses. It is also plausible that some of the hypothetical neuroendocrine genes of interest function by being repressed, rather than induced, upon eIF2α phosphorylation, and therefore only rare gain or alteration of function will be able to suppress the effects conferred by eIF2(αP)-mediated repression of such genes. We have not performed any genetic screens using the starvation response phenotype, which may involve genes that function non-redundantly and specifically in adulthood and would not be associated with the dauer developmental

decision.

A recent study has suggested that mislocalization of DAF-7/TGFβ in the ASI neurons, induced by the presence of misfolded DAF-28(R37C), may be responsible for the *daf-28(sa191)* Daf-c phenotype (Klabonski et al., 2016). Loss of function of *daf-3* that encodes a key downstream effector of the TGFβ pathway, however, has no effect on the dauer entry phenotype (Malone et al., 1996), arguing against a significant contribution of aberrant TGFβ signaling to promoting dauer formation. Furthermore, that *pek-1* mutation exacerbates DAF-7 mislocalization (Klabonski et al., 2016) while suppressing the dauer arrest phenotype indicates that neuronal-eIF2(αP)-mediated dauer entry occurs independently of defects in DAF-7 biosynthesis.

Molecular and genetic basis of sensory integration and dauer formation

While the signal transduction mechanisms regulating dauer formation and maintenance have been extensively studied (reviewed in Hu 2007 and Fielenbach and Antebi, 2008), the molecular and genetic basis of perception and integration of stimuli that trigger the developmental decision remains unclear. In this section, I will discuss my findings concerning neuronal eIF2α phosphorylation and dauer formation specifically in the context of physiological sensory perception and processing that control the diapause decision.

Contributions among dauer-promoting environmental cues

Crowding, as assessed by the presence of the ascarosides/dauer pheromone, starvation and high temperature are stimuli that promote dauer formation (Golden and

Riddle, 1984). The most potent and robust physiological determinant of dauer diapause is the dauer pheromone (Golden and Riddle, 1982), which may reflect ecological functions of the diapause, as dauer larvae are commonly observed under highly populous conditions in rotten fruits (Felix and Duveau, 2012). In contrast to the ascarosides that induce *bona fide* dauers at high frequencies, high temperature alone (27°C), under otherwise optimal growth conditions, induces dauer entry less efficiently and the resultant dauer larvae are paler and recover rapidly (Ailion and Thomas, 2000). While starvation is a sole stressor that induces arrest at the first larval stage (Baugh, 2013), its effect on dauer entry is less defined. Bacterial deprivation in the absence of pheromone has to be administered precisely between the first and second larval stages, the critical period for the dauer decision (Swanson and Riddle, 1981 and Golden and Riddle, 1984b). In our hands, we are only able to induce developmentally arrested larvae, during the presumptive dauer stage, that harbor only some but not all characteristics of the dauer larvae (data not shown).

Elevation of ambient temperature significantly enhances dauer formation in the presence of the dauer pheromone (Golden and Riddle, 1984a and 1984b). Nutrient deprivation is coincident with crowding in plates that are starved out, which promote dauer entry reliably and are used to isolate alleles of the Daf-d genes (Riddle et al., 1981). Similar to dauer entry, dauer recovery also requires assessment of both the crowding and food signals (Golden and Riddle, 1982). Collectively, these observations suggest that nutrient availability and temperature, while on their own are not able to fully mediate the dauer developmental decision, have significant modulating effects on dauer entry mediated by the most critical determinant which is dauer pheromone.

Ascarosides and dauer formation

Because of its robustness, exposure to the dauer pheromone under monoaxenic conditions has become the standard laboratory method to non-genetically induce dauer entry (reviewed in Ludewig and Schroeder, 2013). Both crude pheromone and chemically synthesized ascarosides have been adopted to varying degrees of dauer-promoting efficiency (reviewed in Ludewig and Schroeder, 2013). In virtually all dauer formation assays documented, including those employing highly potent synthetic ascarosides, a tantalizing common requirement to ensure robust dauer formation is the utilization of non-proliferating, usually heat-killed, bacterial food source (Jeong et al., 2005, Kim et al., 2009, McGrath et al., 2011 and Park et al., 2012). The omission of two components conventionally used in worm husbandry, live *E. coli* and peptone, suggest that their nutritional or chemical contributions may alter pheromone perception and sensitivity in the worm, attenuating or counteracting the pro-dauer effects of the ascarosides.

Ascaroside receptors have been identified to function in the ASI and ASK chemosensory neurons (Kim et al., 2009, McGrath et al., 2011 and Park et al., 2012). The ASI and ASK neuron pairs also harbor other chemoreceptors that perceive a wide variety of molecules, which may include metabolites produced by live bacteria that modulate the neuroendocrine and dauer responses. By virtue of their receptor expression patterns, ASI and ASK are equipped with the ability to cell autonomously process sensory inputs that include both the key dauer-regulating cue (ascarosides) and the modulating one (food source).

We hypothesize that the pheromone signal is necessary for dauer formation but

not sufficient on its own, perhaps because the live bacteria provide antagonizing, pro-growth signals. The presence of heat-killed bacteria may remove the nutritional or chemical cues that live bacteria provide to inhibit dauer formation. In the next section, I will discuss how aberrant stress signaling in the sensory neurons may mimic the pro-dauer effects of the absence of live *E. coli* food source.

ASI-specific eIF2α phosphorylation and sensory processing for dauer formation

I have demonstrated that constitutive eIF2α phosphorylation in the ASI neurons bypasses the heat-killed bacteria requirement for the pro-dauer effects of the pheromone (Chapter III, Fig 4B). Specifically, in the presence of the ascarosides and pro-growth live bacteria that inhibit dauer formation, the animals carrying the ASI-specific phosphomimetic eIF2α form dauers substantially, compared to wild-type control (Chapter III, Fig 4B). The dauer-promoting effects are mediated by the same eIF2(αP)-responsive translational regulatory factors described in Chapter III (Chapter III, Fig 4B). Importantly, the *qdIs10* animals do not exhibit a Daf-c phenotype in the absence of the dauer pheromone, indicating that constitutive eIF2α phosphorylation specifically modulates the food-dependent sensitivity to the ascarosides.

In addition to expressing the pheromone receptors, the ASI neuron pair also harbors chemoreceptors that can transduce food availability signals to modulate neuroendocrine production (Bishop and Guarente, 2007, Entchev et al., 2015, Dalfo et al., 2012 and Gallagher et al., 2013). We thus hypothesize that constitutive phosphorylation of eIF2α, a conserved signaling mechanism that is induced by food scarcity, may subdue the pro-growth or nutrition signals from live bacteria in ASI,

mimicking the counteracting, pro-dauer effects conferred by heat-killed bacteria. The effects of eIF2(αP) on ASI may be alterations in the proteome that result in sensitization of the neurons to the pheromone signals or augmentation of pro-dauer neuroendocrine outputs associated with food scarcity. This starvation-like, pro-dauer state of the sensory neurons is reminiscent of the neurons in a previously characterized calcium/calmodulin-dependent protein kinase mutant that has similar aberrant food signal integration, permitting dauer formation even in the presence of ascarosides and anti-dauer live bacteria (Neal et al., 2015).

Recent studies have noted the dramatically distinct effects of live bacteria versus heat-killed bacteria on organismal physiology, including dauer diapause (Neal et al., 2015 and Khanna et al., 2016). While heat-killed bacteria confer pro-dauer effects and impair *C. elegans* growth and reproduction, it remains unclear whether the biological consequences result solely from nutritional deficiency, or that unique chemosensory experiences elicited by the dead microbial environment might be involved. In light of the ancient role of eIF2α phosphorylation in detecting amino acid starvation, it is likely that aberrant eIF2α signaling in ASI may mimic the pro-dauer effects of nutritional scarcity, synergizing with pheromone signaling. Intriguingly, distinct methods used for killing bacteria, including streptomycin (Golden and Riddle, 1982 and McGrath et al., 2011) or UV treatment, differentially influence the sensitivity to the dauer pheromone, potentially dependent on residual metabolic signatures engendered by each method. It is thus possible that aberrant eIF2α signaling in ASI may also in part mimic the pro-dauer effects of microbe-derived chemosensory signals that potentiate pheromone receptivity.

eIF2α phosphorylation and the dauer developmental decision

We have examined the physiological requirements for eIF2α phosphorylation in regulating dauer formation using mutants that fail to phosphorylate eIF2α or to respond to eIF2(αP). Specifically, we examine mutants where *pek-1* or *gcn-2* or all *C. elegans* eIF2α kinase genes are abolished, as well as mutants carrying the unphosphorylatable eIF2α(S49F) variant or the other class II eIF2(αP)-insensitive alterations in *eIF2Bα* or *eIF2γ*. We find that these eIF2(αP)-unresponsive mutants still form dauers when the plates are starved out (Chapter III, Fig S6A), indicating that eIF2α phosphorylation is not the sole requirement for detection of food deprivation in the presence of ascarosides, pointing to non-redundant roles performed by other nutrient-sensing pathways such as AMPK (Cunningham et al., 2014). The eIF2(αP)-insensitive animals also form dauers efficiently, similar to wild-type control, in the presence of the pheromone and heat-killed bacteria, indicating that the pro-dauer nutritional and chemosensory experiences, provided by the absence of live bacteria, can be perceived by other pathways functioning in parallel to modulate ascaroside responsiveness. Collectively, these observations suggest that while aberrant neuronal eIF2(αP)-mediated signaling is sufficient to elicit pro-dauer effects in the presence of the ascarosides, eIF2α phosphorylation functions redundantly with multiple food quantity- and quality-sensing pathways that modulate developmental responsiveness to crowding. While eIF2(αP)-mediated signaling is indispensable for the Daf-c phenotype elicited by neuronal ER stress (Chapter III, Fig 1), under physiological conditions such as when the plates are starved out or in the worm's natural habitats, additional sensory signaling pathways may function, possibly in other cells and tissues, to transduce adverse cues into developmental decisions. We note that

starved-out plates were used for the initial isolation of Daf-d genes (Riddle et al., 1981), hence favoring genes that have critical, nonredundant functions in sensory processing and tissue remodeling required for dauer entry. The characterized Daf-d genes mediating environmental signal integration have therefore largely been those involved in developmental and structural integrity of the sensory structures in the amphid (Albert et al., 1982).

Constitutive neuronal eIF2α phosphorylation and its genetic interactions

Because the *qdIs10* larvae do not form dauer constitutively under optimal growth conditions (abundant live *E. coli* and low population density), constitutive neuronal eIF2α phosphorylation on its own, without the presence of the ascarosides, is not sufficient to promote the dauer decision. On the other hand, the *qdIs10*-mediated dauer entry in response to the dauer pheromone and anti-dauer live bacteria highlights the modulating roles of the aberrant eIF2α status in augmenting the pro-dauer effects of the pheromone. The initial observation that the *qdIs10* animals form dauers on crowded plates where live bacteria are not depleted (Chapter III) can be explained by this modulating nature of ectopic neuronal eIF2(αP), mimicking the dauer-promoting effects contributed by the absence of live bacteria. Because the dauer incidence in the wild-type animals exposed to ascarosides and killed food source is almost 100%, we are unable to reliably assess whether the pro-dauer *qdIs10* can independently further influence the dauer decision.

The modulatory effects of ectopic ASI-specific eIF2α phosphorylation are observed in a few genetic circumstances. As discussed in the first section of this chapter,

the *daf-28(sa191)* Daf-c phenotype is a composite of the predominant pro-dauer eIF2(αP) effects and the more minor contribution by diminished insulin signaling mediated by the DAF-28 absence. The *qdIs10* expression can also enhance the TGFβ receptor mutant Daf-c phenotype (Chapter III, Fig 4A), indicating that eIF2α phosphorylation may function in parallel to the TGFβ pathway which senses both dauer pheromone and food availability. This additive genetic interaction between *qdIs10* and the pheromone-sensitive TGFβ signaling mechanism is reminiscent of the synergistic effects between the neuronal eIF2(αP) that mimics starvation and pheromone treatment. The *qdIs10* expression is only able to modestly enhance the dauer entry phenotype mediated by the insulin receptor mutant (Chapter III, Fig 4A), suggesting that the pro-dauer effects of eIF2α phosphorylation may partially overlap with insulin signaling mechanisms. We note that ASI-specific eIF2(αP) is able to substantially promote dauer formation in an insulin ligand (*daf-28(-)*) mutant (Chapter II, Fig S3), suggesting that the *qdIs10* dauer-promoting effects may be conferred by other insulin peptides or insulin-independent neuroendocrine pathways.

While we have not identified specific neuropeptides conferring the pro-dauer effects of *qdIs10*, we argue that the dauer decision is mediated through a distinct mechanism from general ASI dysfunction. As discussed previously, in the adult stage *qdIs10* expression and genetic ablation of ASI confer distinct phenotypes (starvation response versus normal adulthood). While ablation of ASI constitutively triggers dauer formation despite favorable conditions, the *qdIs10* larvae do not exhibit a Daf-c phenotype in the absence of dauer pheromone. Moreover, while ectopic neuronal eIF2(αP) substantially increases the dauer incidence in the presence of the pheromone

and live bacteria, the completely penetrant level characteristic of strong Daf-c mutations was not observed, underscoring the secondary, modulatory nature of neuronal eIF2α phosphorylation in regulating the dauer decision.

Conclusions

The genetic characterization of the *daf-28(sa191)* dauer developmental decision has revealed the organismal, cell-nonautonomous roles of the PEK-1/PERK branch of the UPR, as well as of the eIF2α phosphorylation translational control mechanism. The forward genetic approach has allowed us to better understand effectors and modifiers of these conserved stress signaling mechanisms at both cellular and organismal levels. Perturbations of proteostasis and translation, particularly in the nervous system, affect multiple aspects of animal physiology, as illustrated by the systemic effects elicited by aberrant eIF2α phosphorylation on larval development and adult stress responses. Alterations in the neuronal eIF2α phosphorylation status may be influenced by nutritional and chemosensory cues specific to the microbial environment, representing a signal transduction mechanism that modulates and optimizes developmental decisions. We anticipate future studies that clarify the nature of the cell-nonautonomous signals transducing the Integrated Stress Response among tissues. A recent study has shown that eIF2α phosphorylation in the skeletal muscle regulates systemic metabolism via secretion of a specific growth factor (Miyake et al., 2016). We speculate that analogous sets of neuroendocrine molecules may mediate the dauer decision and the starvation response elicited by stress-induced eIF2α phosphorylation in the sensory nervous system. The cell-nonautonomous effects of the UPR and eIF2α-mediated translational control illustrate

how multicellular organisms may co-opt conserved stress signaling pathways that maintain cellular homeostasis to modulate or coordinate systemic responses to environmental fluctuations and challenges at the organismal level.

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Table 1: Class III genes

Allele(s)	Molecular identity
pkc-1(qd367)	W113X*
pkc-1(qd366)	C282Y*
pkc-1(qd364)	A545V*
pkc-1(qd368)	splice site (G to A), 5′ to exon 5*
pkc-1(qd365)	splice site (G to A), 5′ to exon 11*
daf-2(qd371)	E1240K**
daf-12(qd372-374)	ND***
sup-1(qd318)	N/A****
sup-2(qd369)	N/A****
sup-3(qd370)	N/A****

*residues and splice sites are based on isoform a of *pkc-1*
** residue is based on isoform a of *daf-2*
***confirmed by complementation testing
*****sup1, sup2 and sup3* may be presented during the dissertation defense and will be further characterized in future studies in the Kim lab