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Report

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

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Summary

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 available online), but the mechanism is not well understood [5–7]. 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

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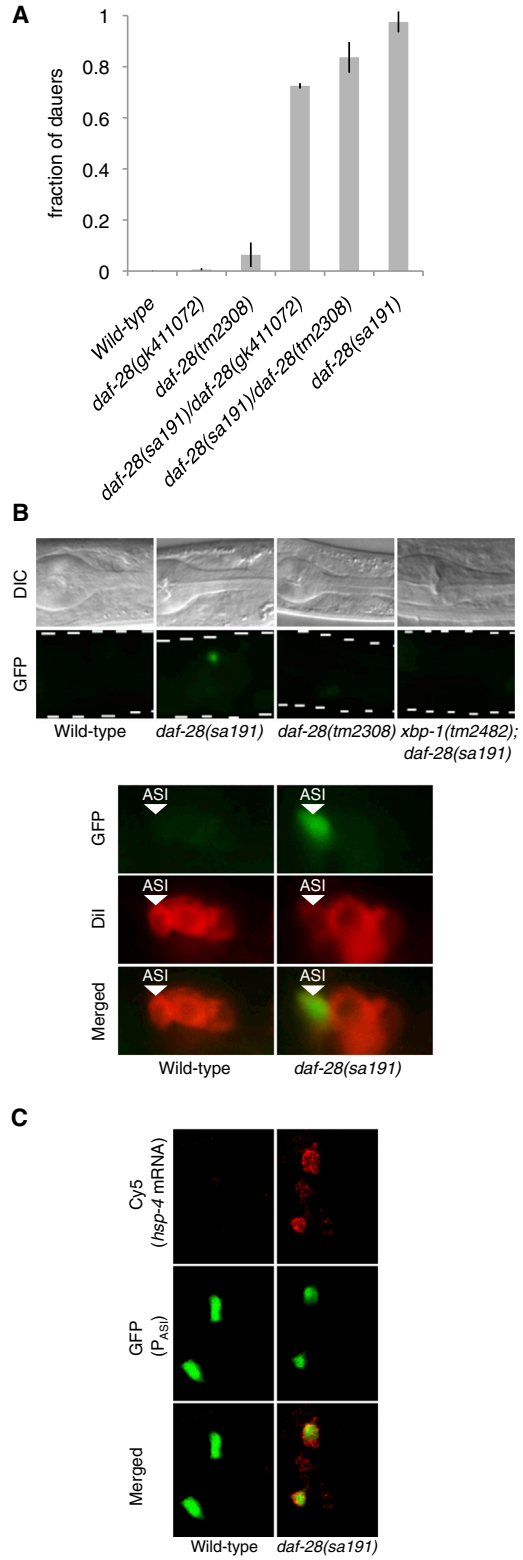

Figure 1. The *daf-28(sa191)* Mutation Causes ER Stress in the ASI Neuron Pair

(A) Fractions of the indicated *daf-28* mutants entering 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. Mean ± SD is plotted. 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). Images shown are representative of three or more independent experiments.

(C) Maximum z projection of single-molecule fluorescence in situ hybridization (smFISH) stacked images of predauer larvae with indicated genotypes. ASI neurons are identified by GFP expression driven by the cell-specific *daf-7* promoter [9, 10].

See also Figures S1 and S2.

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 [15–17]. 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

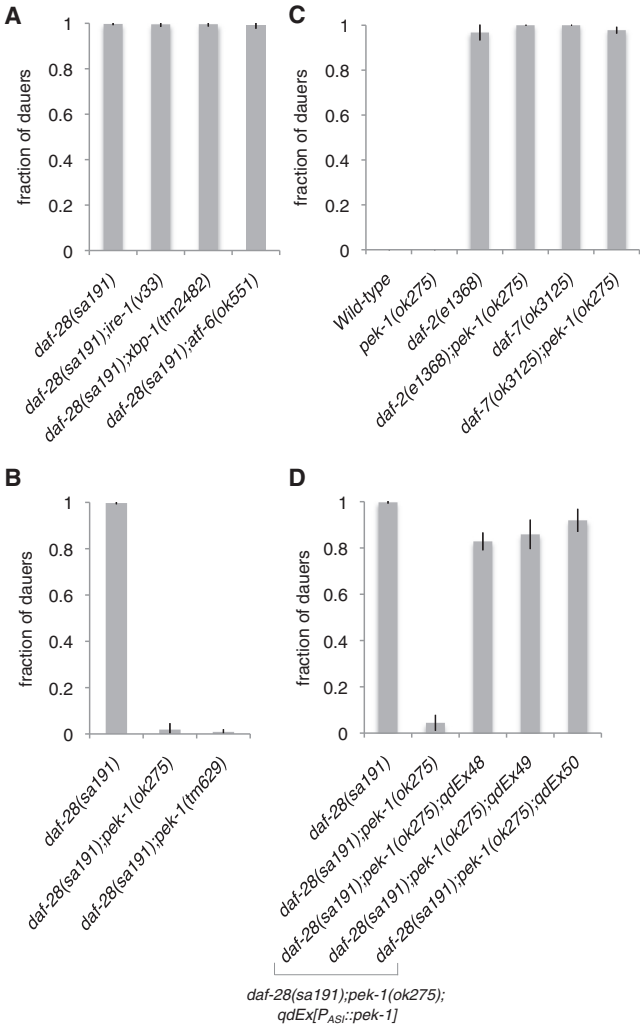

Figure 2. Activation of PERK/PEK-1 in the ASI Neuron Pair of the *daf-28(sa191)* Mutant Promotes the Dauer Developmental Decision

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

(B) Fractions of *daf-28(sa191)* mutants with indicated *pek-1* deficiencies forming dauers at 25°C.

(C) Fractions of the indicated mutants in the *pek-1*-deficient background forming dauers at 25°C.

(D) Fractions of animals with indicated genotypes entering 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).

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

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 [1–4]. 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, 20–23], 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

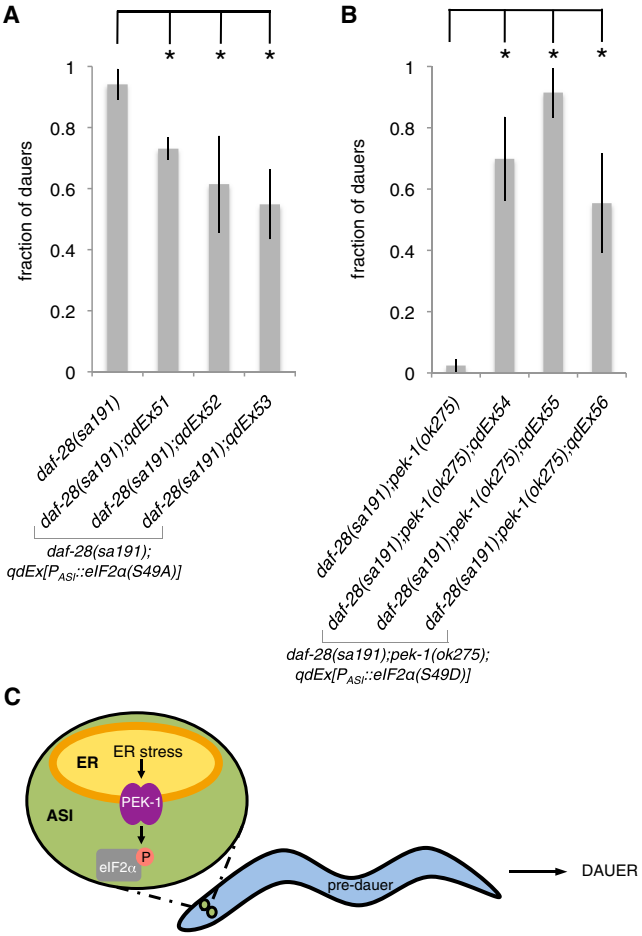

Figure 3. Phosphorylation of Ser49 of eIF2α by PEK-1 in the ASI Neuron Pair Promotes Entry into Dauer Diapause

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

(B) Fractions of animals with indicated genotypes entering the dauer stage at 25°C. The *daf-28(sa191);pek-1(ok275)* strain in this figure represents nontransgenic controls from all three lines. *$p < 0.01$ was determined by paired, two-tailed Student's t test (nontransgenic fraction versus transgenic fraction for each line). Results from the ASI-specific *gpa-4* promoter are shown in this figure (referred to as P_{ASI}). Mean ± SD is plotted. 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.

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, 20–23].

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 [26–28]. 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 described previously [33]. The following strains were used in Figures 1, 2, and 3 (additional strains used in Figures S1, S2, and S3 and Tables S1, S2, and S3 are listed in Supplemental Experimental Procedures): 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(ok275);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]* and ZD951-953 *daf-28(sa191);pek-1(ok275);qdEx54-56[gpa-4p::eIF2α(S49D):: unc-54 3′UTR]*.

Constructs and Generation of Transgenic Lines

Transgenic animals carrying extrachromosomal arrays were generated using standard microinjection methods [34]. Unless otherwise noted, the injection mixture included an indicated PCR-fusion construct (injected at 15 ng/μl) and the *ofm-1p::GFP* coinjection marker (pQZ22, injected at 50 ng/μl), kindly provided by J. Alcedo. PCR fusion was performed as described previously [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. Previous studies have validated the specificity of both promoters (see Figure S3 and Supplemental References)

Dauer 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 3–6 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 hr after the egg-lay midpoint, as at this time point all animals have passed the predauer stages and, for the dauer-constitutive mutants that exit dauer, the dauer larvae have not resumed reproductive development. Dauers were discriminated from nondauers based on radial shrinkage of the body, 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, we used the same position in the incubator in all of the experiments for temperature consistency, and we controlled the population density on each plate by the number of gravid adults laying eggs and the duration of egg lay.

Microscopy

Animals were mounted with 10 mM sodium azide onto slides with a 2% agarose pad. Slides were viewed using an Zeiss AxioImager Z1 fluorescence microscope primarily with a 40×/1.3 oil objective. Fluorescence signals were recorded with a charge-coupled device camera (AxioCam) using constant exposure time without saturation. Images were captured and processed using AxioVision image processor software. The lipophilic dye DiI was used to identify amphid neurons.

Supplemental Information

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.058.

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