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Protein-Repair and Hormone-Signaling Pathways Specify Dauer and Adult Longevity and Dauer Development in *Caenorhabditis elegans*

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Protein damage that accumulates during aging can be mitigated by a repair methyltransferase, the L-isoaspartyl-*O*-methyltransferase. In *Caenorhabditis elegans*, the *pcm-1* gene encodes this enzyme. In response to pheromone, we show that *pcm-1* mutants form fewer dauer larvae with reduced survival due to loss of the methyltransferase activity. Mutations in *daf-2*, an insulin/insulin-like growth factor-1-like receptor, and *daf-7*, a transforming growth factor-β–like ligand, modulate *pcm-1* dauer defects. Additionally, *daf-2* and *daf-7* mutant dauer larvae live significantly longer than wild type. Although dauer larvae are resistant to many environmental stressors, a proportionately larger decrease in dauer larvae life spans occurred at 25°C compared to 20°C than in adult life span. At 25°C, mutation of the *daf-7* or *pcm-1* genes does not change adult life span, whereas mutation of the *daf-2* gene and overexpression of PCM-1 increases adult life span. Thus, there are both overlapping and distinct mechanisms that specify dauer and adult longevity.

Key Words: Adult and dauer life span—Dauer formation—Protein L-isoaspartyl methyltransferase—*daf-2*—*daf-7*.

A molecular rationale for the decreased functional capabilities associated with age is the accumulation of protein damage. Proteins are subjected to a myriad of nonenzymatic covalent modifications that can affect their function (1). L-Aspartic acid and L-asparagine residues are major targets for such reactions that can lead to the predominant formation of L-isoaspartate residues, resulting in a kinked polypeptide chain (2). However, such damaged L-isoaspartate residues are recognized by the protein L-isoaspartyl-*O*-methyltransferase, an enzyme that can initiate their conversion to normal L-aspartate residues in a protein repair reaction (3–8).

The repair methyltransferase has been conserved through evolution, and enzymes from prokaryotes and eukaryotes share a high degree of sequence similarity (9). Genes coding for this enzyme have been found in nearly all of the organisms examined. In *Escherichia coli*, loss of the *pcm* methyltransferase can result in reduced stationary-phase survival (10). In mice, animals lacking the *pcmt-1* enzyme accumulate proteins containing modified aspartyl residues and start to die of uncontrolled seizures right after weaning, with only 50% remaining alive just before they reach sexual maturity (11–14).

In the nematode *Caenorhabditis elegans*, the repair methyltransferase is encoded by the *pcm-1* gene (15). To elucidate the physiological role PCM-1 plays in *C. elegans*, a large portion of the gene was deleted using Tc1-transposon-mediated mutagenesis and excision in an *him-8(e1439)* background (16). The *him-8; pcm-1* double mutant is similar to the control *him-8* animals in morphology, fertility, and adult life span (16). Interestingly, the absence of the enzyme does not appear to cause a marked accumulation of damaged proteins (17). However, *him-8; pcm-1* animals did show two *pcm-1*-specific phenotypes: *pcm-1*-null animals are selected against in long-term, competitive population studies, and they exhibit a shorter dauer life span when compared to *him-8* dauer larvae (16).

The dauer stage is an alternate third larval stage that *C. elegans* will enter under conditions of restricted food supply, elevated temperature, or high pheromone concentration due to a dense population (18,19). This stage is characterized by several changes in the animal: They are thinner and longer than L3 larvae, their cuticle is especially thick, and their intestinal cells appear dark (20,21). Dauer larvae do not feed or defecate due to a constricted pharynx and sealed buccal and anal cavities. These features can contribute to their ability to resist environmental and chemical stresses such as desiccation and sodium dodecyl sulfate (SDS) treatment (20). Dauer larvae survive longer than any other wild-type stage (22). Many mutants have been identified that influence dauer larva formation (21). The phenotype of abnormal dauer larva formation is called Daf. The abnormality can either cause constitutive (Daf-c) entry into the dauer larval stage under conditions where wild-type larvae would grow to reproductive adults or cause defects in dauer larva formation (Daf-d) resulting in

morphologically abnormal, fewer, or no dauer larvae formed under inducing conditions.

To adequately understand the phenotypes of repair methyltransferase deletion mutants in the absence of secondary mutations, the *pcm-1*-null mutation was placed in a wild-type (N2) background. We found the significant phenotypic differences of these mutants in dauer larvae formation as well as longevity. We also found modulation of the *pcm-1* dauer defect by several *daf*-c mutations in the three well-characterized branches of the dauer larva formation pathway (23–27). Mutations in the *daf-7* or *daf-2* genes each increased dauer longevity, whereas only the latter increases adult longevity 25°C, which suggests that some life-limiting processes in dauer larvae and adults are distinct. In addition, deletion of the *pcm-1* gene has no effect on adult life span in either a wild-type or *daf-2* mutant background. Finally, we show that overexpression of the *pcm-1* gene significantly extends adult life span. These results point to a complex interplay of PCM-1 activity with signaling pathways leading to dauer formation and maintenance.

Materials and Methods

Culture Methods and Strains

Animals were maintained on 60-mm Petri dishes containing 10 mL of nematode growth (NG) agar medium seeded with the *E. coli* strain OP50 (28), unless otherwise noted. The temperature-sensitive *daf-c* mutants were cultured at 15°C whereas other strains were grown at 15°C, 20°C, or 25°C. Animals were grown in S Medium, fed OP50, and aerated on a rotary platform shaker (28).

The mutations used in this study were: Linkage Group (LG) I: *daf-16(m26)*; LG III: *daf-2(m41, m596,* and *e1370), daf-7(m62* and *e1372)*; LG IV: *him-8(e1439)*; LG V: *pcm-1(qa201* and *tm363), daf-11(m47).* To generate a single *pcm-1* mutant, the *him-8; pcm-1* strain was backcrossed to N2 five times and polymerase chain reaction (PCR) amplification was used to screen progeny for the *pcm-1* deletion (16,29). The loss of the *him-8* mutation was verified by placing virgin hermaphrodites singly on plates and observing no male progeny from any of the individuals. Double mutants were constructed by mating *pcm-1* males with *daf-c* hermaphrodites (27). F_2 Daf-c dauer larvae were cloned, and their progeny were screened for the *pcm-1* deletion by PCR. After their progeny were collected, the adults were screened for the *pcm-1* mutation by PCR. Several homozygous *pcm-1* single- and double-mutant strains were isolated and behaved similarly.

To obtain transgenic rescue strains of the *pcm-1* gene, the overlapping *pcm-1* and C10F3.4 genes and an approximately 3 kb region upstream of each gene was PCR amplified from *C. elegans* genomic DNA using the Expand Long Template PCR System (Roche, Indianapolis, IN). The primers used were: forward, 5′ TGATCAGTTGGGACCCACCATGAG 3′; reverse, 5′ CTGCAGGAGTGCGGTGCTAATTTC 3′. A 10,853 bp PCR fragment was produced and was cloned into the pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Transgenic lines were obtained by injection of the construct into the gonad (30) of *pcm-1(qa201)* adults along with the transformation marker plasmid containing *myo-3*::GFP. Mutations were introduced into the *pcm-1*/C10F3.4 rescue construct using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). To mutate the methyltransferase active site, the sequence ALDVGSGSG was changed to ALDVGSVSV. The primers used were: forward, 5′ GCTCTTGACGTTGGCTCAGTAAGTGTATATTTGACAGTTTGTATGGC 3′; reverse, 5′ GCCATACAAACTGTCAAATATACACTTACTGAGCCAACGTCAAGAGC 3′. The DNA sequence of the mutagenized clones was confirmed. Transgenic *pcm-1* nematodes containing the mutated clone demonstrated no PCM-1 enzyme activity.

Dauer Life-Span Assay

Synchronous entry into the dauer stage was initiated by crude pheromone in liquid culture with limiting amounts of bacteria. Eggs were isolated from gravid adults by hypochlorite treatment and allowed to hatch overnight at 25°C in the absence of food. These synchronized L1 larvae were counted, and the density was adjusted to 4000 larvae/mL for N2 and *pcm-1* and 2000 larvae/mL for *daf-2, daf-7*, and *daf-2*; *pcm-1*. Induction of dauer formation for N2 and *pcm-1* was by high temperature, high pheromone concentration, and low food concentration, whereas for the *daf-c* strains it was by high temperature and a mutation that signals inappropriate dauer entry in the presence of food alone. N2 and *pcm-1* L1 larvae were placed in S Medium that was a mixture of 25% vol/vol fresh and 75% expended from liquid cultures that had contained growing populations for at least 2 weeks and filter sterilized. Each day, for 3 days, the N2 and *pcm-1* cultures were given a small amount of food such that the cultures would consume the food within a day. The formation of dauer larvae was verified by visual inspection, and these conditions forced nearly 100% of the N2 larvae to enter the dauer stage. The *daf-c* larvae were cultured in fresh S Medium with food and were grown at 25°C.

Nematodes were harvested by sucrose flotation (28). Subsequently, dauer larvae were purified by 1% SDS treatment for 45 minutes at room temperature and washed four times in ice-cold M9. For the dauer life-span assay, animals were maintained in M9 at a density of 2000 dauer larvae/mL with ampicillin at 50 μg/mL and nystatin at 100 μg/mL, and were incubated at either 20°C or 25°C. The cultures were aerated on a platform rotary shaker (150 rpm) and sampled every 5 days. The rate of evaporation was measured by weight loss and was slow, such that nearly all loss was due to sampling. To determine the number alive, three separate samples were removed into sterile glass tubes using glass pipettes because we noted significant adhesion of animals to plasticware. To score at least 30 live larvae per plate, an appropriate volume was spotted on plates using glass micropipettes. The total number of dauer larvae was noted, then the number alive was counted based on movement when touched. At later time points when the samples were concentrated, the total number was based on the average value for the early time points, which were fairly stable. No larvae resumed development and grew in the cultures of aging dauer larvae.

Dauer Formation Bioassay

Isolation of crude pheromone and the dauer formation assay were performed as previously described (31,32), with two exceptions: Streptomycin at 50 μg/mL was added to the plates rather than the bacterial slurry, and only 20 μL of OP50 in M9 (0.05 g cells/mL) was spotted on each plate. Three preparations of pheromone were used in the experiments presented here. Each pheromone preparation was titered using N2. This same wild-type strain was included in each experiment as a control. Eggs laid within 2 hours were cultured at 20°C for 48 hours at which time the developing larvae were scored as predominately L2d and L4 larvae. L4 larvae were removed from the plate. After 24 hours, the L2d larvae that had been left on the plate were scored for formation of dauer larvae. This same protocol was used for epistasis analysis except that the culture temperature was 25°C and no pheromone was added to the standard plate medium.

SDS Sensitivity

Larvae exhibiting dauer morphology were assayed for SDS resistance 96 hours after egg fertilization. Larvae were incubated in a 45 μL drop of 1% SDS in M9 for 45 minutes at room temperature on the lid of a sterile plate (20). The animals were then pipetted to a seeded plate. The number of larvae in the spot was scored, and the animals were allowed to recover at 15°C or 20°C, depending on the strain. Two to four days later, the number and larval stage of the live animals were scored. The number dead was also determined. Dead animals were the size of dauer larvae, did not move from the spot, and did not move when touched with a platinum wire pick.

Brood Size and Adult Life-Span Assays

The reproductive capacity was determined at 20°C. Individual adults were moved every 12 hours during the fertile period to a new plate with a thin bacterial lawn to aid in egg counts (27). Eggs were counted on each plate and then allowed to develop and counted again as L4 larvae. The life-span assays were conducted at 25°C, and Day 1 is the first day of adulthood (27). The animals were scored daily, excluding animals that had internal hatchlings, herniated gonads, or were lost.

Results

Deletion of the pcm-1 *Gene Does Not Affect Fertility or Viability*

The effects of the *pcm-1* deletion were previously assayed in a strain containing a mutation in the *him-8* gene, which results in a high incidence of male progeny from adult hermaphrodites. This *him-8(e1439); pcm-1(qa201)* strain showed a reduced brood size compared to N2 wild-type animals but a similar brood size to that of the *him-8(e1439)* control (16). Subsequent large-scale RNA interference (RNAi) experiments suggested that interfering with *pcm-1* expression may cause 14% embryonic lethality (33). To dissect *pcm-1* phenotypes from *him-8* phenotypes and to detect the possible low levels of embryonic lethality, we backcrossed the *him-8; pcm-1* strain to the N2 wild-type strain and generated a *pcm-1(qa201)* single mutant. We found the brood size and hatching efficiency of *pcm-1 (qa201)* to be 274 ± 73 ($n = 16$) and 96% ± 2 ($n = 8$), respectively. This is not significantly different from the brood size of 286 ± 47 ($n = 16$) and hatching efficiency of 94% ± 5 ($n = 12$) for N2 at 20°C. Thus, deletion of *pcm-1* does not reduce the number or viability of the eggs produced.

Reduced Life Span of pcm-1 *and Increased Life Span of* daf-7 *and* daf-2 *Dauer Larvae*

Previous work noted a reduced median survival of 24.5 days for *him-8; pcm-1* dauer larvae compared to 27 days for *him-8* dauer larvae (16). These median survival times are substantially shorter than the 45 days first reported for N2 wild type (22). To dissect the *pcm-1* phenotypes from *him-8* phenotypes, we examined the single mutant *pcm-1(qa201)* and compared it to N2. In addition, this allowed for comparisons to the previously published data on wild-type dauer longevity (22). In both of the previous experiments, animals were allowed to form dauer larvae as populations increased and food was depleted over a 10- to 14-day period (16,22). Here, survival may have been artificially increased if any of the dauer larvae resumed development and produced progeny. In our experiments, entry into the dauer stage was tightly controlled by pheromone treatment of synchronized L1 larvae, and all larvae entered the dauer stage within a 12-hour period. Complete absence of food during the dauer survival assay prohibited recovery and growth to reproductive adults thereby maintaining age synchrony.

In our synchronized-entry dauer life-span assay, the survival of N2 dauer larvae at 20°C was similar to that previously observed with 50% survival at 40 days (compared to 45 days) and with a maximal survival of 75 days (compared to 80 days) (Figure 1A) (22). As expected, *pcm-1(qa201)* dauer larvae showed reduced survival, but they displayed even shorter life spans than those described previously (Figure 1A) (16). Dauer larvae with a deletion in the *pcm-1* gene had a median life span only one quarter of the N2 dauer life span at 20°C. At the beginning of the assay, the larvae were treated with SDS to select for only the dauer larvae, because dauer-like or younger larvae are killed by this treatment. All *pcm-1* dauer larvae were dead by day 40, whereas 50% of N2 dauer larvae remained alive at that time. These results indicate that PCM-1 plays a major role in survival of dauer larvae.

Mutations in the *daf-7* and *daf-2* genes cause inappropriate formation of dauer larvae and increased survival as starved L1 larvae, but only *daf-2* adults show a robust life-span extension at 25°C (23,34). It was unknown whether these mutations also affect dauer survival. We found that two *daf-c* mutations, *daf-2(m41)* and *daf-7(e1372),* extend dauer life span (Figure 1A). This is the first extension of dauer survival to be observed. The median survival for these strains is 53 days. In light of the different effects on adult life span, it is interesting that the dauer life span was similar for *daf-7* and *daf-2*. After 40 days of age, the increase in survival of *daf-c* mutant dauer larvae relative to N2 dauer larvae becomes statistically significant with *daf-7(e1372)*

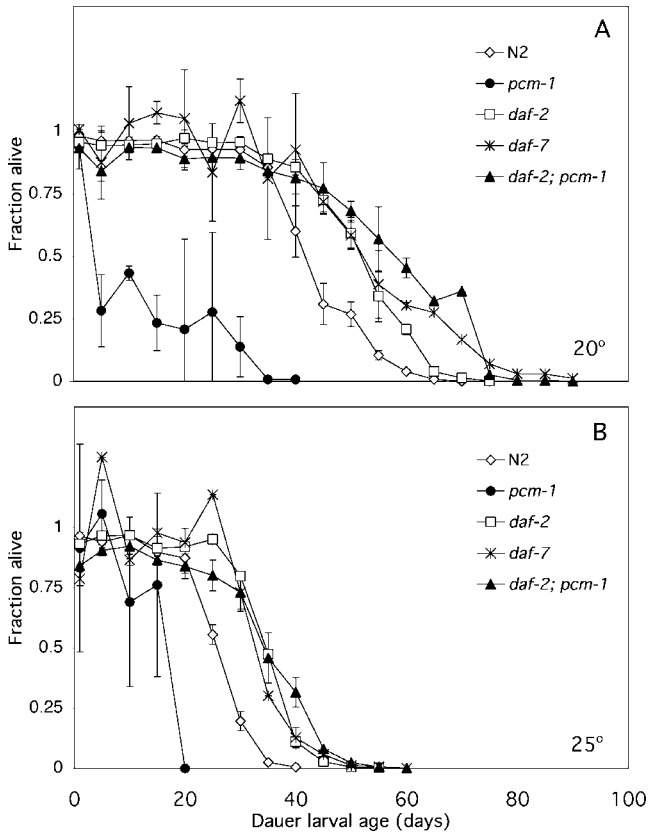

Figure 1. Dauer life span at different temperatures. **A,** Survival curves at 20°C. **B,** Survival curves at 25°C. N2 (*open diamonds*), *pcm-1(qa201)* (*filled circles*), *daf-2(m41)* (*open squares*), *daf-7* (*cross hatch*), and *daf-2(m41); pcm-1(qa201)* (*filled triangles*). Error bars indicate standard deviation from three replicate samples of the total population. Similar relative survival curves were obtained in replicate experiments.

surviving the longest at 20°C. The survival of *daf-2(m41); pcm-1* is similar to the survival of *daf-2(m41),* which is increased compared to N2. The median survival of *daf-2(m41); pcm-1* larvae at 20°C is 58 days. Therefore, *daf-2(m41)* suppressed the life-span defect of *pcm-1*-null mutant dauer larvae. These *daf-c* dauer larvae results suggest differences in the life-limiting processes in dauer larvae and adults.

As poikilotherms, many processes in *C. elegans*, such as growth and adult longevity, are affected by temperature (35,36). When we assayed dauer life span at a higher temperature, we found that the metabolically depressed dauer larvae do respond to temperature. There is a substantial decrease in life span for all genotypes at 25°C (Figure 1B). The median survival for wild-type dauer larvae was only 27 days at 25°C compared to 40 days at 20°C. The median survival for *daf-7* at 25°C is 33 days, which is also reduced from that observed at 20°C. The median survival of 35 days for the *daf-2* and *daf-2(m41); pcm-1* dauer larvae show that they are still long-lived at 25°C, compared to N2. The maximum life span for *pcm-1* mutant dauer larvae was reduced by half. This short dauer life-span phenotype is suppressed by a mutation in the *daf-2* gene. The dramatic reduction in dauer life span observed with a 5° increase in cultivation temperature is surprising, considering the perception that the dauer stage is an unchanging stage that is resistant to environmental insults.

Table 1. *pcm-1*-Null Mutants Show Reduced Numbers of Dauer Larvae in Response to Pheromone at 20°C

Genotype	Percent Dauer
Experiment 1: 20 μL of Pheromone per Plate	
N2	38.5 ± 9.8 (240)
pcm-1(qa201)	9.4 ± 8.1 (263)*
daf-16(m26)	0.0 ± 0.0 (208)†
daf-2(m41)	99.2 ± 1.4 (236)†
daf-2(m41); pcm-1(qa201)	41.6 ± 6.3 (223)‡
Experiment 2: 60 μL of Pheromone per Plate	
N2	96.4 ± 2.4 (358)
him-8(e1489)	93.3 ± 1.9 (470)
him-8(e1489); pcm-1(qa201)	59.6 ± 16.2 (143)§

Notes: Values presented are the mean percentage of dauer larvae per plate ± standard deviation. Three plates per genotype were assessed, and the total number of animals for each genotype is in parentheses. Another *pcm-1*-null isolate also yielded significantly fewer dauer larvae than N2 in response to pheromone (data not shown).

*$.005 < p < .05$ when compared to N2 (Student's *t* test).

†$p < .005$ when compared to N2.

‡$p < .005$ when compared to *daf-2(m41).*

§$.01 < p < .05$ when compared to *him-8(e1489)* control.

Deletion of the pcm-1 *Gene Inhibits Dauer Formation in Response to Pheromone*

To understand the reduced survival of *pcm-1* mutant dauer larvae, we first tested for a possible defect in dauer formation. Wild-type animals that sense pheromone and have a limited food supply will enter into the dauer stage (19). We found that *pcm-1* mutants formed fewer dauer larvae in response to pheromone relative to N2 larvae (Table 1). With 20 μL of pheromone per plate at 20°C, *pcm-1* formed only 24% of the dauer larvae formed by N2. We saw no dauer larva formed by Daf-d *daf-16(m26)* larvae (negative control) and observed defects in dauer formation at different concentrations of pheromone (Table 1, Figure 2) (23). In addition, we analyzed the *him-8*; *pcm-1* strain for the Daf-d phenotype using 60 μL of crude pheromone. The *him-8* mutation does not appear to affect dauer formation because the result for the single mutant is indistinguishable from N2 in response to pheromone. However, the double mutant *him-8*; *pcm-1* formed only 62% of the dauer larvae formed by the single mutant *him-8* at this high pheromone concentration (Table 1). We were also able to confirm the effect of the *pcm-1* mutation on dauer formation using a second deletion allele, *pcm-1(tm363)* (Table 2). Finally, we performed a rescue experiment by preparing transgenic worms with a wild-type clone of the *pcm-1* locus in the *pcm-1* deletion strain. We also prepared transgenic worms with a mutated clone of the *pcm-1* gene where two glycine residues, which are essential for enzymatic activity, were changed to valine residues. No methyltransferase activity could be detected in the mutant transgenic worms where activity was present in the wild-type transgenic worms (data not shown). Transgenic larvae were then tested in a pheromone-induced dauer formation assay. Comparison of the control transgenic strain, PL50, and a strain carrying a wild-type clone of the *pcm-1* locus,

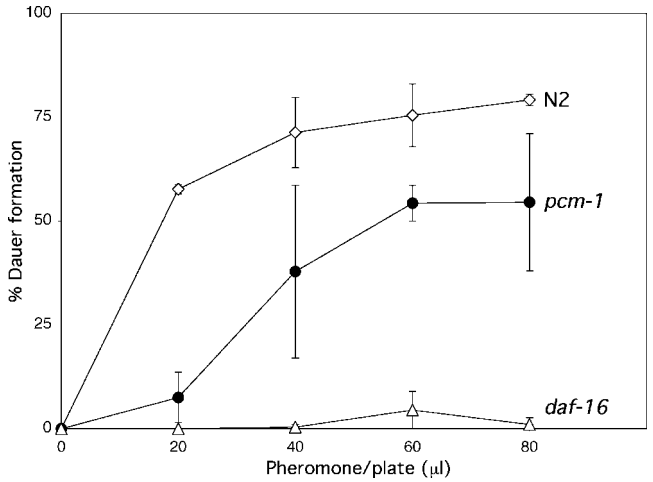

Figure 2. Dauer formation with pheromone. Dauer formation for N2 (*open diamonds*), *pcm-1(qa201)* (*filled circles*), and *daf-16(m26)* (*open triangles*) at 20°C with increasing amounts of pheromone per plate. Error bars indicate standard deviation from three replicate cohorts.

Table 2. Dauer Formation in Response to Pheromone at 20°C of *pcm-1* Deletion Mutants and Transgenic Strains

Genotype	Percent Dauer
40 μL of pheromone per plate	
N2	22.7 ± 4.1 (204)
pcm-1(qa201)	11.5 ± 7.6 (153)
pcm-1(tm363)	5.7 ± 5.8 (150)*
PL50 *pcm-1(qa201); luEx21[myo-3::GFP]*	5.8 ± 3.6 (176)*
PL51 *pcm-1(qa201); luEx22[pcm-1 + myo-3::GFP]*	28.1 ± 15.8 (370)
PL54 *pcm-1(qa201); luEx25[pcm-1(G88V G90V) + myo-3::GFP]*	14.1 ± 4.7 (362)
PL55 *pcm-1(qa201); luEx26[pcm-1(G88V G90V) + myo-3::GFP]*	3.8 ± 4.1 (361)†

Notes: A wild-type plasmid rescues the *pcm-1* mutant defect. A plasmid with two mutated residues in the PCM-1 active site shows reduced dauer formation and thereby does not rescue. Values presented are the mean percentage of dauer larvae formed per plate ± standard deviation. Three plates per genotype were assessed, and the total number of animals for each genotype is in parentheses. A different preparation of pheromone was used here than in the experiment described in Table 1.

*$.005 < p < .05$ when compared to N2 (Student's t test).

†$p < .005$ when compared to N2.

PL51, shows substantially more dauer larvae in the latter (Table 2). We conclude that this clone rescues the dauer formation defect of *pcm-1(qa201)*. This rescue depended on the transgene having the wild-type *pcm-1* sequence; animals with the mutant transgene (PL54 and PL55 strains) formed fewer dauer larvae than did animals with the wild-type transgene (PL51 strain) (Table 2). These results clearly demonstrate the importance of an active PCM-1 methyltransferase in dauer formation.

It is possible that the *pcm-1* larvae are deficient in sensing pheromone. To test this, we used a *daf-7::GFP* fusion transgene whose expression in ASI neurons is suppressed by the presence of pheromone (37). The transgene *mIs5[rol-6 (su1006) daf-7p::GFP]* was crossed into *pcm-1(qa201)*, and the resulting strain did express green fluorescent protein (GFP) appropriately under reproductive growth conditions. The ASI neurons fluoresced brightly in the absence of both pheromone and food in 14 of 14 *mIs5* larvae and in 21 of 21 *mIs5; pcm-1* larvae. However, GFP fluorescence was not detected in the ASI neurons in the presence of pheromone and the absence of food in 9 of 9 *mIs5* larvae and in 23 of 23 *mIs5; pcm-1* larvae. Thus, deletion of the *pcm-1* gene does not affect responsiveness to pheromone for the down-regulation of DAF-7::GFP synthesis.

We then analyzed the interaction of the *pcm-1* and the *daf-2* genes with pheromone induction of dauer formation because *pcm-1* larvae respond poorly and some *daf-c* mutations result in animals that are more sensitive to pheromone than N2 (32). We found that *daf-2(m41)* formed transient dauer larvae with no pheromone at 20°C, and formed nearly 100% dauer larvae with only 5 μL of pheromone per plate at 20°C (data not shown). In the double mutant, we found that the *daf-2* mutation enables *pcm-1*-null mutants to form more dauer larvae than the single *pcm-1* mutant in response to pheromone (Table1). However, the *pcm-1* mutation reduces the ability of *daf-2* mutants to be sensitized to form dauer larvae by pheromone (Table 1). These results suggest that animals with the *daf-2(m41)* allele, like N2 animals, depend on PCM-1 function to reliably execute dauer formation in the presence of pheromone at 20°C.

SDS Sensitivity of pcm-1 *Dauer Larvae*

The formation of dauer-like or partial dauer larvae has been described for mutations in other *daf-d* genes; these larvae have abnormalities in specialized dauer structures including the alae, lateral hypodermal cells, and the pharynx, as well as sensitivity to 1% SDS (24,38). We examined the *pcm-1* dauer larvae alae and lateral hypodermal bodies by differential interference contrast microscopy, and no obvious differences were observed. Measurements of the pharynx were made, and we detected no differences in the maximal width of the anterior bulb (metacarpus), the maximal width of the terminal bulb and isthmus, or the length from the buccal cavity to the grinder. Thus, there are no gross morphological differences in the *pcm-1* dauer larvae.

N2 dauer larvae survive 1% SDS treatment and, after removal from SDS and upon feeding, resume development to reproductive adults (20). The *pcm-1* larvae picked for SDS treatment were thin and long, did not forage, appeared not to pump, had dark intestinal cells, and displayed typical dauer posture. The majority of these apparent dauer larvae lacking the *pcm-1* gene, however, are highly sensitive to SDS treatment (Table 3). In these experiments, we measured sensitivity 96 hours after egg fertilization; dauer larvae tested after 72 hours showed a reduced sensitivity (data not shown). In each experiment, the percentage of *pcm-1* dauer larvae killed by SDS treatment is much higher than the percentage of N2 dauer larvae killed. This defect is also noted in the original *pcm-1*-null strain, *him-8*; *pcm-1*, which has a significantly higher percentage of larvae killed by SDS treatment than the *him-8* mutant control (Table 3). Because we find no obvious defects in the alae, lateral hypodermal cells or the pharynx, the *pcm-1* mutation does not appear to cause morphological defects that lead to SDS sensitivity.

Table 3. Sodium Dodecyl Sulfate (SDS) Sensitivity of Dauer-Like Larvae

Genotype	Percent Dead
Experiment 1	
N2	0.8 ± 0.9 (287)
pcm-1(qa201)	78.8 ± 8.2 (214)*
Experiment 2	
N2	1.7 ± 2.0 (268)
pcm-1(qa201)	42.9 ± 17.4 (309)*
Experiment 3	
N2	5.0 ± 2.8 (305)
him-8 (e1489)	5.8 ± 3.0 (279)
PL12 *pcm-1(qa201)*	68.1 ± 10.5 (280)*
PL13 *pcm-1(qa201)*	74.0 ± 16.0 (199)*
him-8; pcm-1	65.8 ± 22.0 (61)†

Notes: Values presented are the average percentage of dauer larvae killed by SDS treatment ± the standard deviation with the number of animals in parentheses. The average represents three replicate trials.

*$p < .005$ compared to N2 (Student's t test).

†$p < .005$ compared to *him-8(e1489)* control.

Table 4. Percent Dauer Larvae and Other Stages of Double Mutants with a *daf-c* Mutation and the *pcm-1*-Null Mutation at 25°C

Genotype	% Dauer	% L3, L4, Adult	% L1, L2	*N*
N2	0.0	99.3	0.7	718
pcm-1(qa201)	0.0	98.8	1.2	818
daf-2(m41)	97.1	0.0	2.9	766
daf-2(m596)	92.4	0.0	7.6	1065
daf-2(e1370)	98.1	0.0	1.9	720
daf-7(m62)	100.0	0.0	0.0	924
daf-7(e1372)	99.0	0.0	1.0	717
daf-11(m47)	83.3	15.9	1.7	723
daf-2(m41); pcm-1	98.7	0.0	1.3	635
daf-2(m596); pcm-1	87.6	0.0	12.4*	942
daf-2(e1370); pcm-1	81.3	0.0	18.7†	670
daf-7(m62); pcm-1	99.5	0.0	0.5	847
daf-7(e1372); pcm-1	99.9	0.0	0.1	1101
daf-11(m47); pcm-1	90.7	6.9	2.3	652

Notes: Values presented are the mean percentage of animals in each stage per plate with three plates for each genotype at 25°C. Animals that crawled off the agar and desiccated were excluded. Similar results were obtained in replicate experiments.

*$.005 < p < .05$ when compared to the single mutant (Student's t test).

†$p < .005$ when compared to the single mutant.

In the process of analyzing the transgenic strains, another defect was noted in *pcm-1* larvae. The plates were checked daily for 10 days rather than scoring them once. Initially, the pheromone plates had 41–119 dauer larvae and, for plates with *pcm-1* animals, 0–9 individuals were observed to recover from the dauer stage each day, whereas no wild-type dauer larvae resumed growth. Some *pcm-1* individuals remained as dauer larvae for at least 10 days. The recovery was not synchronous and variable in number, as previously observed for other *daf* mutants (25). The *pcm-1* dauer larvae that inappropriately resume development in the presence of dauer-inducing levels of pheromone may be the individuals that were scored as SDS-sensitive.

Epistasis Analysis of pcm-1 *for Dauer Larvae Formation*

It is possible to order gene function into genetic pathways by analyzing the phenotype of double mutants whose single mutants have opposite phenotypes. Numerous such epistasis studies have been carried out with mutations that affect dauer formation (23–27). Here, we tested the functional relationships between *pcm-1* and *daf-2, daf-7,* and *daf-11*. Mutations in the *daf-2* gene lead to multiple temperature-sensitive phenotypes: Daf-c, embryonic arrest, morphological differences, increased adult life span, and late progeny production (23,34,39,40). The *daf-2* gene encodes an insulin-like receptor tyrosine kinase (40). Mutations in the *daf-7* gene, which shows homology to transforming growth factor-beta (TGF-β), cause temperature-sensitive constitutive dauer formation (23,37). Mutations in the *daf-11* gene, encoding a guanylyl cyclase, also cause temperature-sensitive constitutive dauer formation (24,41). Finally, larvae with a mutation in the *daf-12* gene are Daf-d, and these mutations suppress the Daf-c defect of *daf-7* and interact with particular alleles of *daf-2* (23–27). The *daf-12* gene shows homology to nuclear hormone receptors (42,43). In wild-type larvae under growth conditions, the inferred order of gene function places the TGF-β-like *daf-7* and the guanylyl cyclase *daf-11* pathways both functioning to inactivate the *daf-12* gene, thereby preventing dauer formation. The insulin-like signaling *daf-2* pathway prevents dauer formation by inactivating the *daf-16* gene.

To order *pcm-1* function relative to other genes in the dauer formation pathway, the phenotype of double mutants was analyzed. In this case, dauer formation was induced at 25°C by *daf-c* mutations in the absence of pheromone. Appropriately, N2 and the *pcm-1*-null mutant did not form dauer larvae at this temperature, whereas all of the *daf-c* mutants did form dauer larvae (Table 4). No *daf-7*; *pcm-1* or *daf-2*; *pcm-1* double-mutant larvae grew to L3, L4, or adult at the restrictive temperature similar to their *daf-c* single-mutant controls. Similarly, loss of the *pcm-1* gene did not affect the ability of *daf-11(m47)* mutant animals to enter the dauer stage, resembling previous studies of this allele (24). We conclude that the *pcm-1* deletion does not suppress the Daf-c phenotype of *daf-2, daf-7,* or *daf-11*. There is a statistically significant increase in the pre-dauer larval arrest for two of the *daf-2; pcm-1* double mutants (Table 4). The dauer formation observed here for the *daf-2(m41)*; *pcm-1* larvae appears to be contrary to the results of the pheromone assay, but here the dauer-formation cues were temperature and the *daf-2* reduction-of-function mutation. Lack of PCM-1 does not inhibit dauer formation of these *daf-c* mutations; therefore, PCM-1 is not required for these mutants to enter the dauer stage.

We extended our analyses by determining the SDS sensitivity of the dauer larvae formed in the *daf-c; pcm-1* double mutants (Table 5). Mutations in the *daf-2* gene increase the percentage of SDS-resistant dauer larvae formed in *pcm-1*-null animals to different extents depending on the *daf-2* allele. The *daf-2(m41)* allele is able to suppress the dauer SDS sensitivity phenotype to a greater extent than *daf-2 (e1370)* allele, which shows a stronger suppression than the *daf-2(m596)* allele. With regard to dauer formation, the

Table 5. Sodium Dodecyl Sulfate (SDS) Sensitivity of *daf-c; pcm-1* Double-Mutant Dauer Larvae

	Percent Dead *pcm-1* Genotype	
daf-c Genotype	*pcm-1(+)*	*pcm-1(qa201)*
daf-2(m41)	2.4 ± 2.4 (122)	16.4 ± 10.5 (127)
daf-2(e1370)	1.6 ± 1.4 (125)	36.7 ± 11.2 (120)*
daf-2(m596)	1.7 ± 1.5 (118)	68.0 ± 5.7 (125)†
daf-7(m62)	2.0 ± 1.7 (106)	97.5 ± 2.5 (118)†
daf-7(e1372)	25.2 ± 8.5 (119)	100.0 ± 0.0 (97)†
daf-11(m47)	5.7 ± 3.0 (105)	100.0 ± 0.0 (85)†

Notes: Dauer larvae formation was induced by the *daf-c* mutation in response to 25°C. Values given are the average percentage of 1-day-old dauer larvae killed by SDS treatment ± standard deviation of three replicate plates with the number of dauer larvae treated given in parentheses. Animals were scored 48 hours after SDS treatment. Similar results were obtained in replicate experiments.

*$.005 < p < .05$ when compared to the single mutant (Student's *t* test).

†$p < .005$ when compared to the single mutant.

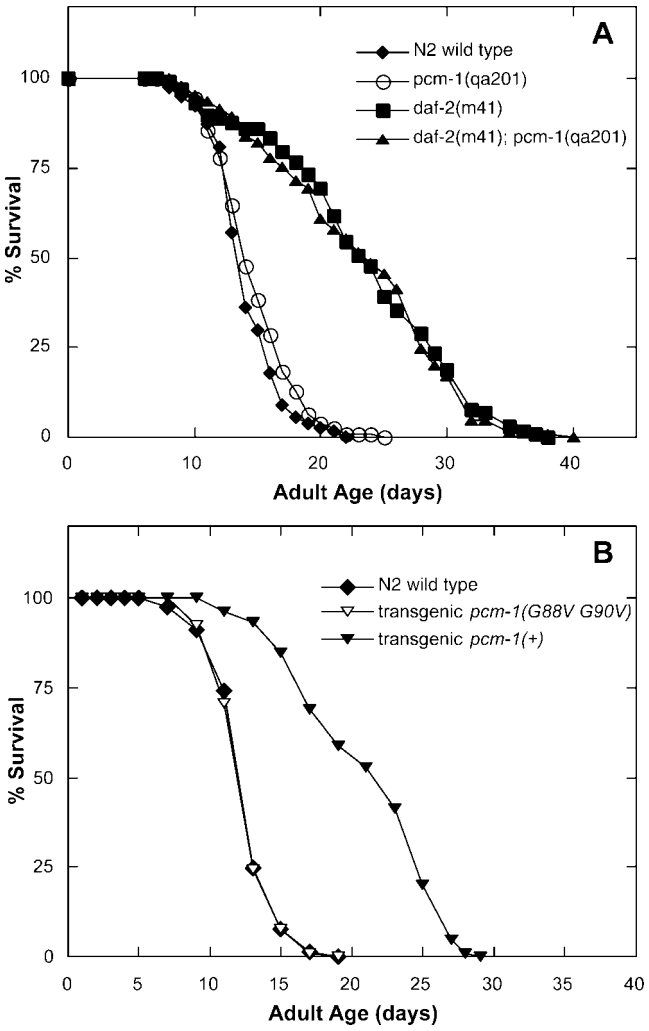

Figure 3. Adult hermaphrodite life span assayed at 25°C. **A,** N2 (*filled diamonds*), *pcm-1(qa201)* (*open circles*), *daf-2(m41)* (*filled squares*), and *daf-2(m41); pcm-1(qa201)* (*filled triangles*). L4 animals were selected at Day 0 and molted to adults within several hours. **B,** N2 (*filled diamonds*), transgenic overexpression of *pcm-1(+)* (*filled triangles*), and inactivated transgenic *pcm-1(G88V G90V)* (*open triangles*). Transgenic L2 larvae were selected by green fluorescent muscles from *myo-3::GFP*. Day 0 is the day on which the animals were L4 larvae, and they molted to adults within several hours.

m596 allele displays a more severe defect than the other two alleles (39). The partial suppression observed for the formation of SDS-sensitive dauer larvae of *pcm-1* mutants is probably due to use of non-null alleles of *daf-2* that likely have residual activity. The *daf-2(m41)* missense mutation, which suppresses the *pcm-1* phenotypes to the greatest extent, is in the cysteine-rich region of the ligand-binding domain (44). The *m596* mutation is in the ligand-binding domain but downstream of the cysteine-rich region (45). The *e1370* mutation is in the kinase domain (40). The results could be interpreted to mean that *pcm-1* is upstream of *daf-2* for dauer maintenance. Alternatively, the most severe allele tested displayed both phenotypes in *daf-2(m596); pcm-1* because the predominant class of animals constitutively form SDS-sensitive dauer larvae. In this case the interpretation is that *pcm-1* most likely functions in parallel to *daf-2* for dauer maintenance.

Mutations in both the *daf-7* and *daf-11* genes fail to suppress the formation of SDS-sensitive dauer-like larvae due to the *pcm-1* deletion (Table 5). In fact, in *daf-7; pcm-1* and *daf-11; pcm-1* animals nearly all of the dauer larvae formed are SDS sensitive, whereas in *pcm-1*-null animals, at most three quarters of the larvae were observed to be SDS sensitive (Table 2). Thus, the *pcm-1* is epistatic to *daf-7* and *daf-11*. We interpret these data to suggest that PCM-1 functions in parallel or downstream of DAF-7 and DAF-11 in dauer morphogenesis or commitment to remain as a dauer.

Deletion of the pcm-1 *Gene Does Not Affect Adult Hermaphrodite Life Span in Either a Wild-Type or* daf-2 *Mutant Background*

To test the hypothesis that the protein repair activity of the *pcm-1* gene contributes to adult survival at the elevated temperature of 25°C where more protein damage would be expected to occur, we assayed the wild type and four single mutant isolates of *pcm-1*. Overall, we noted no statistically significant decrease in adult life span of *pcm-1* deletion mutants compared to N2 (Figure 3, Table 6). In one experiment, a statistically significant increase in life span was computed for three of the four isolates tested. However, this trend was not observed when the experiment was repeated (Table 6). The failure to reproduce the slight, statistically significant life-span increase reinforces the practice of replicate testing with more than one isolate to confirm life-span differences.

We then considered that more time might be required for nematodes to accumulate a level of protein damage that would negatively impact life span. Mutations in the *daf-2* gene result in adult life-span extension (34). Therefore, a double mutant with the *pcm-1(qa201)* deletion and the *daf-2 (m41)* mutation was tested to establish whether an animal with an adult life span nearly twice that of N2 requires PCM-1 to mitigate the negative consequences of protein damage over the extended life span. Surprisingly, the adult life spans at 25°C of *daf-2(m41)* animals and the double

Table 6. Adult Survival Statistics

Strain and Genotype*	Mean Adult Life Span (Days)	Last Quartile (days)	*N*
Experiment 1			
N2 (+)	15 ± 0.3	16 ± 0.4	147
PL12 *pcm-1(qa201)*	17 ± 0.4‡	19 ± 0.6	109
PL13 *pcm-1(qa201)*	16 ± 0.4†	18 ± 0.6	108
PL14 *pcm-1(qa201)*	16 ± 0.4‡	19 ± 0.5	107
PL15 *pcm-1(qa201)*	15 ± 0.3	16 ± 0.5	119
Experiment 2			
N2 (+)	14 ± 0.2	16 ± 0.3	158
PL12 *pcm-1(qa201)*	14 ± 0.2	16 ± 0.3	159
PL15 *pcm-1(qa201)*	15 ± 0.3†	17 ± 0.4	151
daf-2(m41)	24 ± 0.7	29 ± 0.7	128
PL16 *daf-2(m41); pcm-1*	22 ± 0.6	28 ± 0.5	141
PL17 *daf-2(m41); pcm-1*	23 ± 0.6	28 ± 0.5	130
Experiment A			
N2 (+)	13 ± 0.3	14 ± 0.4	71
PL54 *pcm-1(qa201); luEx25[pcm-1(G88V G90V) + myo-3::GFP]*	14 ± 0.4	16 ± 0.4	37
PL51 *pcm-1(qa201); luEx22 [pcm-1 + myo-3::GFP]*	24 ± 0.5‡	28 ± 0.6	115
Experiment B			
N2 (+)	13 ± 0.2	13 ± 0.2	146
PL54 *pcm-1(qa201); luEx25[pcm-1(G88V G90V) + myo-3::GFP]*	13 ± 0.2	13 ± 0.2	132
PL51 *pcm-1(qa201); luEx22 [pcm-1 + myo-3::GFP]*	21 ± 0.4‡	25 ± 0.4	129

Notes: Values given are ± standard deviation for each group at 25°C.

*Independent isolates are distinguished by their strain names.

†$.005 < p < .05$ compared to N2 by the Logrank (Mantel–Cox) method, using Statview software.

‡$p < .005$ compared to N2 by the Logrank (Mantel–Cox) method.

mutant *daf-2(m41)*; *pcm-1(qa201)* were similar (Figure 3, Table 6). Thus, *pcm-1* is not required for *daf-2(m41)* adult longevity.

Overexpression of the pcm-1 *Gene Increases Adult Life Span*

The transgenic strains created to establish that the *pcm-1* deletion was responsible for the dauer defect contain many copies of the *pcm-1* gene. By quantitative reverse transcription-PCR, a 20-fold increase in messenger RNA level was found in the transgenic strain PL51, which verified overexpression of *pcm-1*. To test whether overexpression of PCM-1 has an effect, we determined the adult life span of transgenic animals. We assayed adults from both strains carrying wild-type *pcm-1* and strains carrying enzymatically defective *pcm-1* transgenes. As before, we conducted the adult life-span experiment at the elevated temperature of 25°C, expecting more protein damage to occur. A substantial increase in adult life span was observed for adults overexpressing the *pcm-1* gene (Figure 3B, Table 6). No statistically significant difference in adult life span was detected for transgenic adults carrying a defective *pcm-1* compared to N2 (Figure 3B, Table 2). The similar longevity for these two genotypes demonstrates that the exposure to ultraviolet light as larvae, to select transgenic animals, did not affect adult life span. The observed longevity suggests that there is a benefit of excess PCM-1, even though there is no evidence for survival defects in adult animals lacking PCM-1.

Discussion

We show here that the lack of the PCM-1 protein repair methyltransferase in *C. elegans* has little or no effect on embryogenesis, non-dauer larval development, reproductive capacity, adult longevity, or gross morphology. In contrast, overexpression of PCM-1 increases adult life span. The lack of PCM-1 does cause significant defects in dauer formation. *pcm-1* mutant larvae respond poorly to a pheromone signal as indicated by the reduced number of dauer larvae formed. Many of the apparent dauer larvae that do form are sensitive to SDS treatment and therefore are not true dauer larvae. This sensitivity may be due to a lack of commitment to the dauer state in the presence of pheromone, as shown by the tendency of dauer larvae to form transiently. Alternatively, PCM-1 may be important in dauer morphogenesis itself, such as a role in cuticle formation and/or maintenance of the dauer state. Additionally, the dauer larvae that are initially SDS resistant fail to survive for extended periods. In contrast to *pcm-1* dauer larvae, we find that *daf-2* and *daf-7* mutant dauer larvae survive longer than wild-type dauer larvae. Unexpectedly, a 5° decrease in temperature doubles survival time for dauer larvae, whereas a 10° decrease is necessary to double adult survival time. The genetic and environmental modulation of dauer larval survival noted here sheds light on the survival properties of dauer larvae in general.

The effect of the loss of the PCM-1 methyltransferase on dauer larva, the stage specialized for stress resistance and long-term survival under unfavorable conditions, is consistent with results from other organisms. In bacteria, the loss of the *pcm* gene has no apparent effect in log-phase growth but significantly reduces the survival of stationary phase cells under environmental stresses (10). In mice, repair-methyltransferase deficient pups have seizures and die a premature death shortly after weaning (13,14,46,47). Interestingly, adult life span is extended in *Drosophila* that overexpress the *Pcmt* methyltransferase when cultured at 29°C, but not when the flies are cultured at 25°C, illustrating that the methyltransferase becomes important to the survival of the animal under stress (48). This appears to be the case in *C. elegans* as well.

The *pcm-1*-null mutants cannot remain as dauer larvae as well as wild type. This observation distinguishes *pcm-1* Daf-d from *che* and *osm* mutants that have defects in chemosensory neurons and thereby fail to form dauer larva in response to pheromone (21,32,49). The *pcm-1* response to increasing concentrations of pheromone resembles wild-type larvae lacking ASJ neurons, which results in fewer dauer larvae formed in response to pheromone (50). The ASI neuronal marker (*daf-7::GFP*) is appropriately regulated in response to pheromone in *pcm-1*-null mutants. PCM-1 may have a role in processing information in pheromone-sensing

neurons or in formation of the non-ASI pheromone-sensing neurons. It is also possible that the integration of sensory cues in the absence of PCM-1 favors reproductive growth by default. In dauer larva formation, the decision whether to form a dauer larva or grow to an adult is not equally weighted, in that the decision to grow is irreversible and the decision to form a dauer larva is continually reassessed. In the *pcm-1* mutant case, the sensory cues appear to be received and transduced to signal initiation of dauer morphogenesis. However, many of the *pcm-1* mutants fail to remain committed to the dauer state.

Dauer formation of *pcm-1* mutants at 20° in the presence of pheromone was modulated by *daf-2*. The *daf-2(m41)* mutation allows *pcm-1*-null mutants to form more dauer larvae in response to pheromone. Coincidently, the *pcm-1* mutation inhibits the ability of *daf-2(m41)* to form as many dauer larvae as the *daf-2* single mutant. Because both the *daf-2* and *pcm-1* phenotypes are observed in the double mutant, it may be inferred that these genes appear to function in parallel pathways. However, because the mutant *daf-2* allele used is not a null allele and probably has residual activity, the observation is also consistent with *daf-2* acting downstream of *pcm-1*, with *pcm-1* negatively regulating *daf-2*.

The *daf* genes have been ordered by analysis of epistasis data into parallel TGF-β–like, insulin-like, and cyclic guanosine monophosphate signaling pathways that converge in the process of dauer formation (23–27). We performed epistasis analysis with *pcm-1* to understand its function in the context of the classic dauer formation pathways. In our epistasis analysis with the *daf-c; pcm-1* double mutants, we found that all the larvae attempt to form dauer larvae in response to the internally generated signal due to a *daf-c* mutation in any of the three signaling pathways. Therefore, the *pcm-1* deletion does not suppress the Daf-c phenotype of *daf-2, daf-7,* or *daf-11*. Both mutant phenotypes are observed in the *daf-c; pcm-1* double mutants because SDS-sensitive dauer-like larvae are constitutively formed. We found that mutations in *daf-7* and *daf-11* do not suppress the SDS sensitivity of *pcm-1* mutant dauer larvae and actually increase the penetrance of dauer-like larvae formation. Similar genetic interactions with mutations in *daf-2* and *daf-7* have been previously noted for *rop-1,* leading to the conclusion that this gene has general effects on the dauer formation process (51). This could also be the case with *pcm-1*. Based on previous reports, the genetic interactions of *pcm-1* are reminiscent of *daf-9*. One interpretation of our results, considering a classic Daf-d phenotype, is that *pcm-1* functions in parallel or downstream of *daf-7* and *daf-11* and in parallel or upstream of *daf-2*. However, in the studies with transgenic strains we observed inappropriate exit from the dauer stage, which might be considered transient dauer formation. If inability to remain dauer is the primary defect, then another interpretation would be that *pcm-1* does not function directly in the decision to initiate dauer formation, as do *daf-2, daf-7,* and *daf-11*, but rather in the decision to remain dauer.

The predicted biochemical outcome of the absence of PCM-1 is increased accumulation of damaged protein. Such accumulation was not detected previously in aged *pcm-1* dauer larvae, so degradation of L-isoaspartyl–containing proteins by active proteolytic pathways may occur, because no biochemical evidence suggests the presence of another L-isoaspartyl-*O*-methyltransferase in *C. elegans* (17). Alternatively, if the methyltransferase has a limited cellular distribution, it may be difficult to detect changes in whole animal lysates arising from repair in the PCM-1–expressing cells. However, aged dauer larvae have been shown to accumulate other types of damage that is reversed upon exit of dauer and resumption of development (52). It is possible that L-isoaspartyl damage accumulates with age in proteins responsible for the development and maintenance of dauer larvae and is at the root of the inappropriate decision to resume development in the presence of pheromone in the *pcm-1* mutants. Another possibility is that the incomplete proteolysis of L-isoaspartyl–containing proteins leads to fragments that can compromise the function of these or other cells (14,53). PCM-1 may function to ensure the complete proteolytic digestion of interfering or unneeded proteins during dauer formation, considering the increased need due to autophagy-dependent tissue remodeling that is crucial for dauer formation (54,55). Finally, PCM-1 may have functions in dauer maintenance that are independent from a role in protein damage repair. We speculate that this role involves a neuroendocrine function that is shared between the various neurons that regulate dauer formation via different signal transduction pathways (50). This role may be the common aspect between nematodes and mice, because neuronal function is most affected in mice lacking PIMT (7).

Finally, the observation of the enhanced life span of mutant dauer larvae in this work is novel. The increased dauer life span of the two *daf-c* genotypes, *daf-2* and *daf-7*, was similar. This result differs from the adult Age phenotype because *daf-7* adults are not long-lived, at least at 25°C (27,56). At 20°C in the presence of fluorodeoxyuridine, it has recently been reported that there is an increase in the adult life span of *daf-7* mutants (57). Enhanced longevity of *daf-7* and *daf-2* dauer larvae may result from the altered entry of the larvae into the dauer stage, physiological differences of the *daf-c* dauer larvae, or a combination of the two. Instead of entering dauer by sensing high pheromone and low food, temperature-sensitive *daf-c* mutations induce dauer larva formation while feeding and thus may have more energy stores initially. Alternatively, there may be differences in the gene expression and metabolism of *daf-c* dauer larvae from that of wild-type pheromone-induced dauer larvae. That there is overlap in the genes that regulate adult life span and dauer life span has been known for some time. However, as in the case of *pcm-1* and *daf-7,* a novel observation is that there are also genes that have no effect on adult life span, but influence dauer life span. The *pcm-1* dauer larvae may be impaired in long-term survival because they inappropriately resume development. In the dauer survival assay, there is no pheromone and no food, so even if a dauer larva attempted to resume development, lack of food would prevent growth. In essence, we suggest that the *pcm-1* dauer larvae are short-lived because they initiate an energetically demanding process and starve to death. The results presented here demonstrate that longevity can depend on the proper cellular response to external signals.

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