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Defective responses to oxidative stress in protein L-isoaspartyl repair-deficient *Caenorhabditis elegans*

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Abstract

We have shown that *Caenorhabditis elegans* lacking the PCM-1 protein repair L-isoaspartyl methyltransferase are more sensitive to oxidative stress than wild-type nematodes. Exposure to the redox-cycling quinone juglone upon exit from dauer diapause results in defective egg laying (Egl phenotype) in the *pcm-1* mutants only. Treatment with paraquat, a redox-cycling dipyridyl, causes a more severe developmental delay at the second larval stage in *pcm-1* mutants than in wild-type nematodes. Finally, exposure to homocysteine and homocysteine thiolactone, molecules that can induce oxidative stress via distinct mechanisms, results in a more pronounced delay in development at the first larval stage in *pcm-1* mutants than in wild-type animals. Homocysteine treatment also induced the Egl phenotype in mutant but not wild-type nematodes. All of the effects of these agents were reversed upon addition of vitamin C, indicating that the developmental delay and egg-laying defects result from oxidative stress. Furthermore, we have demonstrated that a mutation in the gene encoding the insulin-like receptor DAF-2 suppresses the Egl phenotype in *pcm-1* mutants treated with juglone. Our results support a role of PCM-1 in the cellular responses mediated by the DAF-2 insulin-like signaling pathway in *C. elegans* for optimal protection against oxidative stress.

Keywords

oxidative stress; *Caenorhabditis elegans*; protein L-isoaspartyl O-methyltransferase (*pcm-1*); delay in development; egg-laying defect (Egl)

1. Introduction

Reactive oxygen species are produced as by-products of normal metabolism and can directly damage proteins and other cellular components. Cells protect themselves with a variety of antioxidants and detoxification enzymes (Halliwell and Gutteridge, 2007). The expression of these enzymes in response to oxidative stress in *Caenorhabditis elegans* is primarily initiated by the insulin-like signaling pathway mediated by the DAF-2 receptor (Honda and Honda, 1999; Gami and Wolkow, 2006; Murakami, 2007). Down-regulation of the DAF-2 pathway up-regulates the activity of DAF-16, a transcription factor that promotes the expression of anti-oxidant enzymes (Ogg et al., 1997; Braeckman et al., 2001; Murphy et al., 2003; McElwee et al., 2003; Chávez et al., 2007). Mutations in the pathway can give up to a ten-fold increase in

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lifespan (Ayyadevara et al., 2008). In addition to the DAF-2 pathway, the mitogen-activated protein kinase (MAPK) pathway also responds to environmental stress and regulates development and lifespan by interacting with DAF-16 (Wolf et al., 2007). *C. elegans* mutants that are hypersensitive to the increased generation of reactive oxygen species exhibit premature aging (Yanase et al., 2002). For example, the *mev-1* mutant, which lacks a functional cytochrome b subunit of complex II in the electron transport chain, has a shorter lifespan in comparison to its wild-type counterpart (Ishii et al., 1993; Yamamoto et al., 1996).

Another type of protein damage during aging occurs by the non-oxidative deamidation and isomerization of asparaginyl and aspartyl residues. This damage can be recognized by the protein repair L-isoaspartyl *O*-methyltransferase, which initiates the conversion of isomerized aspartyl residues to normal L-aspartyl residues (Clarke, 2003). Interestingly, oxidative damage can increase the load of isomerization damage in cells. For example, increased levels of L-isoaspartyl residues are found in erythrocyte hemoglobin (O'Connor et al., 1988) and membrane proteins (Ingrosso et al., 2000) exposed to oxidative stress, as well as in erythrocytes from glucose-6-phosphate dehydrogenase deficient-patients that are more susceptible to oxidative damage (Ingrosso et al., 2002). Additionally, radiation-induced reactive oxygen species were found to induce protein isomerization in cultured human cells that was reversed with antioxidants (D'Angelo et al., 2005). Furthermore, endothelial cells lacking the protein repair methyltransferase are more susceptible to apoptosis induced by hydrogen peroxide treatment, whereas cells overexpressing the enzyme are more resistant (Cimmino et al., 2008). Finally, proteins in mouse brain that most rapidly accumulate isoaspartyl damage can also be prone to oxidative damage (Zhu et al., 2006). Hence, a connection between oxidative stress and protein isomerization damage and repair has been proposed. We were thus interested in understanding the convergence of the oxidative stress response and protein isomerization/repair pathways.

In *C. elegans*, the protein L-isoaspartyl *O*-methyltransferase enzyme is encoded by the *pcm-1* gene. *Pcm-1* deletion mutant *C. elegans* show normal adult lifespan and no defects in development or fecundity when grown under standard conditions (Kagan et al., 1997; Banfield et al., 2008). However, mutant nematodes are at a competitive disadvantage when co-cultured with wild-type animals (Kagan et al., 1997). Significant effects have been seen in *pcm-1* mutant nematodes under environmental stress: the survival of L1 larvae under starvation conditions is reduced in the mutants (Gomez et al., 2007). *Pcm-1* mutant nematodes also show defects in the dauer phase (Banfield et al., 2008; Gomez et al., 2008). Under unfavorable conditions, such as low food supply and oxidative stress, *C. elegans* enter dauer diapause, an alternate third larval stage in which nematodes are more resistant to environmental stress; as dauer larvae, they have SDS-resistant cuticles and enhanced expression of enzymes involved in resistance to oxidative stress (Cassada and Russell, 1975; McElwee et al., 2003). *C. elegans* lacking *pcm-1* tend to form fewer dauer larvae (Banfield et al., 2008) and have a reduced lifespan in the dauer stage (Kagan et al., 1997; Banfield et al. 2008; Gomez et al., 2008). In addition, *pcm-1* dauer larvae show a reduction in autophagy (Gomez et al., 2007). Interestingly, the *pcm-1* mutant defect in dauer survival is reversed when the *daf-2* gene is also mutated (Banfield et al., 2008). Finally, PCM-1 overexpression leads to adult lifespan extension in *C. elegans* (Banfield et al., 2008) and *Drosophila* (Chavous et al., 2001). These results suggest an interplay between PCM-1 activity and DAF-2 signaling and their joint participation in the regulation of lifespan and resistance to stress.

In this paper, we show that *pcm-1* mutant *C. elegans* are more sensitive to oxidative stress than their wild-type counterparts. Following exposure to the redox-cycling agent juglone, only *pcm-1* mutant dauer larvae entered the adult stage with a defect in egg laying. Nematodes containing a mutation in both *pcm-1* and insulin-like receptor *daf-2* genes no longer displayed the Egl phenotype. The bipyridyl compound paraquat induced a delay in development in wild-

type and *pcm-1* nematodes that is most prominent at the second larval stage in the mutant nematodes. Upon exposure to homocysteine, an amino acid derivative that both inhibits methylation reactions and induces oxidative stress, wild-type and *pcm-1* mutant *C. elegans* underwent a delay in development that was more severe in the absence of PCM-1. All of the phenotypes that developed as a result from treatment with these three oxidizing agents were prevented upon addition of the antioxidant vitamin C, suggesting that the effects were oxidative stress-induced. We hypothesize that PCM-1 may function to oppose the actions of the DAF-2 mediated pathway not only to enhance survival under unfavorable conditions, but also to enhance resistance to oxidative stress.

2. Experimental procedures

2.1. Worm strains and culture conditions

Standard procedures used to maintain *C. elegans* strains were adapted from Sulston and Hodgkin (1988). M9 media and S media were prepared as described (Lewis and Fleming, 1995). The N2 strain was obtained from the *Caenorhabditis* Genetics Center (St. Paul, MN). The *pcm-1* (*qa201*) mutant lacks exons 2–5 of the *pcm-1* gene and alternative exon 1 from the partially overlapping *C10F3.4* gene (Fig. 1; Kagan et al., 1997; www.wormbase.org). This allele was backcrossed eight times into an N2 background (Banfield et al., 2008). The *pcm-1* (*tm363*) strain was a gift from Shohei Mitani at Tokyo Women's Medical University in Tokyo and lacks exons 1–2 of the *pcm-1* gene and regions of the first intron of the *C10F3.4* gene (www.wormbase.org). The *C10F3.4* deletion mutant *tm2679* lacks exons 6–9 of the *C10F3.4* gene and was also a gift from Shohei Mitani at Tokyo Women's Medical University. *daf-2* mutants in linkage group III include *daf-2(m41)*; *daf-2(e1370)*; *pcm-1(qa201)*; and *daf-2(m596)*; *pcm-1(qa201)*. Two transgenic strains (*PL51* and *PL54*; Banfield et al., 2008) were also used. Strain *PL51* expresses a plasmid encoding PCM-1 in a *pcm-1* mutant background, resulting in an approximate seven-fold overexpression of the methyltransferase over wild-type N2 worms grown in liquid culture (Supplemental Table S1). Strain *PL54* contains a similar plasmid with two mutated residues in the PCM-1 active site (*G88V G90V*) and demonstrates no methyltransferase activity (Supplemental Table S1). *Daf-2* strains were maintained at 15 °C or 20°C on nematode growth medium plates streaked with OP50 *Escherichia coli* (NGM +OP50); other strains were similarly maintained but at 20 °C (Sulston and Hodgkin, 1988).

2.2. Preparation of eggs and dauer larvae

Individual preparations of about 100 eggs were made by mixing approximately 20 egg-laying adults in 0.7 ml of M9 medium, 0.25 ml of 5.6 % sodium hypochlorite, and 0.05 ml of 10 M sodium hydroxide for 2 min at room temperature. The eggs were pelleted by centrifugation and resuspended in 0.1 ml of S medium and were then immediately plated on 60 mm plastic Petri dishes containing about 7 ml of NGM agar that had been previously streaked with 1 mg of *E. coli* OP50 cells (wet weight) and incubated for 24 h at room temperature. For preparation of dauer larvae, eggs were transferred to 35 mm plates as described above but that were seeded with half the number of *E. coli* cells and that also contained pheromone. Pheromone was prepared by organic extraction of S media used to grow large cultures of nematodes that had exhausted their OP50 food source (Golden and Riddle, 1984). Plates were incubated for 72 h at 20 °C. Dauer larvae were manually picked for transfer either to plates containing paraquat or homocysteine derivatives or to drops containing juglone (see below).

2.3. Determination of developmental stage

The larval stage was determined by monitoring the development of the vulva under a dissecting microscope and was confirmed by body length (Lewis and Fleming, 1995). The Egl phenotype was determined by observing the distinguishing features of a swollen abdomen containing unhatched eggs coupled with decreased locomotion (Trent et al., 1983, Bastiani and Mendel

2006). Dauer larvae were characterized by their body length and width, decreased motility, absence of pharyngeal activity, and dark shaded abdomens (Lewis and Fleming, 1995).

2.4. Treatment of eggs and dauer larvae with juglone

Eggs (about 100) were plated on 60 mm NGM+OP50 plates containing 236 μ M juglone at 20 °C and were scored for survival on an hourly basis by their ability to advance to L1 larvae. For the effects on dauer larvae, eggs (about 100) were transferred to five freshly-made 35 mm NGM+OP50 pheromone plates. The plates were sealed with Parafilm and incubated at 20 °C for 72 h when dauers had formed. For each strain, a group of 50 dauers was manually transferred to a 30 μ l drop of S medium on the lid of the plate that either contained no addition, 236 μ M juglone, 0.5 mM or 1.0 mM L-vitamin C, or combinations of juglone and vitamin C. Juglone (5-hydroxy-1,4-naphthoquinone, Sigma Chemicals # H47003) was made as a 10 mM stock in ethanol; vitamin C (L-ascorbic acid) was a Fluka product (# 95209). The dauer larvae were allowed to incubate in the drop for 90 min at room temperature and were subsequently transferred to two 60 mm NGM+OP50 plates using glass capillary tubes. Dauer larval survival was monitored after 24 h, 48 hr, and 72 hr by their response to mechanical stimulation. The presence of the egg-laying defect (Egl) was determined after plating on NGM+OP50 plates.

2.5. Treatment with paraquat

Paraquat (methyl viologen, Sigma Chemicals # 856177) and/or vitamin C were added to liquid NGM agar just prior to pouring on 60 mm plates. After the agar solidified, plates were streaked with 1 mg wet weight of OP50 *E. coli* cells and were incubated overnight at room temperature. The following day, approximately 100 eggs were added to the plate which was then sealed with Parafilm and incubated at 25 °C for 48 h before determination of the developmental state. Adult survival was determined as described in Supplemental Table S2.

2.6. Treatment with homocysteine and homocysteine derivatives

DL-Homocysteine was obtained from Sigma Chemicals (# H4628), DL-homocysteine thiolactone hydrochloride was obtained from ICN Biomedicals (# 101976), and DL-homocystine was obtained from Sigma Chemicals (# H0501). These derivatives and/or vitamin C were added to liquid NGM agar just prior to pouring on 60 mm plates. After the agar solidified, plates were streaked with 1 mg (wet weight) of OP50 *E. coli* cells and were incubated overnight at room temperature. The following day, approximately 100 eggs were added to the plate which was then sealed with Parafilm and incubated at 25 °C for 48 h before determination of the developmental state. Adult survival was determined as described in Supplemental Table S2.

3. Results

3.1. The oxidizing agent juglone induces Egl egg-laying defects in pcm-1 mutant dauer larvae

Juglone is a quinone that is taken up by cells and reduced to the semiquinone radical by NAD (P)H flavoenzymes such as cytochrome P450. The semiquinone radical can react with molecular oxygen to form superoxide anion and to reform the quinone for additional cycles of superoxide generation and diversion of reduced nicotinamides from their role in maintaining reduced glutathione. In addition, the semiquinone can reduce iron from Fe³⁺ to Fe²⁺, a species that can subsequently react with hydrogen peroxide (formed from superoxide by the dismutase) to generate destructive hydroxyl radicals via Fenton chemistry (Halliwell and Gutteridge, 2007). In *C. elegans* studies, juglone has been used to select for oxidative stress-resistant mutants, partially because it can penetrate the cuticle structure (De Castro et al., 2004). Because of the links between oxidative damage and protein isomerization damage, we were interested

in asking if nematodes lacking the PCM-1 protein repair L-isoaspartyl methyltransferase might be particularly sensitive to juglone treatment.

Two distinct *pcm-1* deletion strains, *qa201* and *tm363*, were used in the oxidative stress experiments. The *pcm-1* deletion strain *qa201* lacks exons 2–5 of the *pcm-1* gene, whereas *pcm-1* deletion mutant *tm363* lacks exons 1–2 (Fig. 1). No protein L-isoaspartyl methyltransferase activity is detectable in either mutant strain (Supplemental Table S1). The *pcm-1* deletion mutants *qa201* and *tm363* also lack portions of the overlapping gene *C10F3.4*; specifically, *pcm-1* deletion mutants *qa201* and *tm363* lack *C10F3.4* exon 1 and *C10F3.4* intronic regions, respectively (Fig. 1). To determine if oxidative stress phenotypes were specific to the *pcm-1* deletion, the *C10F3.4* deletion strain *tm2679*, lacking exons 6–9, was also tested.

Initially, young adults from the two *pcm-1* deletion strains were treated with 236 μ M juglone in liquid culture or on NGM plates containing juglone using methods described previously (Cypser and Johnson, 2002; De Castro et al., 2004). We observed no difference in the survival of the *qa201* or the *tm363 pcm-1* mutants and the wild type N2 nematodes under these conditions (Supplemental Table S2). We also tested the ability of eggs from each of these strains to undergo larval development in both liquid cultures containing juglone and juglone-containing (236 μ M) NGM+OP50 plates. However, under our conditions, eggs from wild-type and *pcm-1* mutant strains did not hatch to first stage larvae (L1) animals.

We then examined whether dauer larvae from each strain could withstand juglone treatment. Dauer larvae from both wild-type and *pcm-1* mutant *C. elegans* strains were treated with 236 μ M juglone for 90 min and were monitored for development and changes in morphology after transfer to plates containing a food source and lacking juglone. Under these conditions, wild-type dauer larvae remained healthy, developed normally, and laid eggs that developed into healthy young larvae. However, the *pcm-1* mutant dauer larvae were affected (Fig. 2). As shown in Table 1, upon exposure to juglone, approximately 28% and 24% of the of the *pcm-1* (*qa201*) and *pcm-1* (*tm363*) dauer larvae developed into defective egg-laying adults. The egg-laying phenotype (Egl) is characterized by reduced motility, egg retention, and a swollen abdomen containing late-developed larvae (Trent et al., 1983; Desai and Horvitz, 1989; Bastiani and Mendel, 2006). The juglone treated *pcm-1* mutant worms displayed all of these phenotypes, as well as an abnormal pharynx and intestine (Fig. 2). Additionally, the adult nematodes retain eggs that have developed past the stage at which they are normally expelled through the vulva; as a consequence, the egg-laying defective animals appear more transparent than normal gravid adults (Fig. 2; Desai and Horvitz, 1989).

To confirm that juglone-mediated oxidative stress was the cause for the Egl phenotype in the *pcm-1* mutants, dauer larvae were incubated in droplets containing both juglone and the antioxidant vitamin C. At 1 mM vitamin C, the Egl defect was prevented in both *pcm-1* mutant strains (Table 1). Hence, juglone-induced oxidative stress results in defects in egg laying in animals lacking PCM-1 activity.

To demonstrate that the Egl defect in the *pcm-1* mutant strains was not due to the loss of the *C10F3.4* product, we repeated these experiments with the *tm2679 C10F3.4* deletion strain. Only a small, statistically insignificant, number of Egl nematodes resulted from treatment of *C10F3.4* mutant dauer larvae with juglone (Table 1), suggesting that it is the loss of the methyltransferase that is responsible for the Egl defect. We also measured the adult survival and egg viability of the *tm2679* strain treated with juglone but observed no differences when compared to the N2 wild-type strain (Table 1).

To determine if the sensitivity of *pcm-1* dauer larvae to juglone was mediated by changes in the DAF-2 signaling pathway, we then asked whether an additional mutation in the insulin-

like receptor *daf-2* gene suppresses the defects displayed by the *pcm-1* mutant dauer larvae under oxidative stress conditions. Dauer larvae from the *daf-2(m41)*, *daf-2(e1370);pcm-1(qa201)*, and *daf-2(m596);pcm-1(qa201)* strains were treated with juglone as previously described and their development was monitored for 48 hours. Unlike the *pcm-1* mutant dauer larvae, a majority of the *daf-2(e1370);pcm-1(qa201)* and *daf-2(m596);pcm-1(qa201)* mutant dauers did not display the egg-laying defect and instead developed into gravid, egg-laying adults (Table 2). The progeny were healthy and did not display any defects in motility and development. Thus, the additional mutation in the *daf-2* receptor suppressed the defects in egg laying displayed by the *pcm-1* mutant dauer larvae exposed to oxidative stress. This result is consistent with PCM-1 exerting its effect by acting upstream of DAF-2 or in a parallel pathway, perhaps in the same way previously shown for dauer formation and longevity (Banfield et al., 2008). This result also provides additional support towards the hypothesis that PCM-1 may be working alongside the insulin-like signaling pathway to regulate cellular responses to stress in *C. elegans*.

3.2. The oxidizing agent paraquat induces a delay in development that is more severe in *pcm-1* mutants

We next examined the effects of paraquat to compare the oxidative stress phenotypes induced by a distinct oxidizing agent. Paraquat is a redox-cycling agent that also generates reactive oxygen species. Following entry into the cell, paraquat accepts electrons from iron-sulfur proteins and flavins associated with the electron transport chain and is reduced to the bipyridyl radical. The bipyridyl radical may then reduce molecular oxygen to generate superoxide, which can be converted to hydrogen peroxide by superoxide dismutase and peroxiredoxins (Halliwell and Gutteridge, 2007). Unlike the situation described above with juglone where adults died within 2–5 h, treatment with paraquat induces a delay in development and reduced fecundity in *C. elegans* (Ishii et al., 1993; Hartman et al., 1995; Fujii et al., 2005). We thus wanted to compare the effects of paraquat with juglone on protein repair-deficient nematodes. We first treated adult wild-type and *pcm-1* mutant nematodes (both *qa201* and *tm363*) with 0.1 mM and 0.2 mM paraquat. We found no significant effect on survival rate of wild-type animals, confirming the results of previous studies (Ishii et al., 1993; Hartman et al., 1995; Fujii et al., 2005). Additionally, we found no effect of the *pcm-1* mutation upon survival after paraquat treatment (data not shown).

However, we observed a significant delay in development in all strains treated with these concentrations of paraquat and a greater delay in *pcm-1* mutant animals, resulting in larger populations of the first and second larval stages (Fig. 3; panels A and B). Upon treatment with 0.1 mM paraquat, 3.5% and 6.3% of the *pcm-1* mutant animals delayed at the L1 and L2 stages, respectively, in comparison to the 0% and 2% of the wild-type animals. In 0.2 mM paraquat, 7.5% and 40.5% of the *pcm-1* mutants were arrested at the L1 and L2 stages, compared to 5.2% and 3.6% of the wild-type animals. The remaining animals from both wild-type and *pcm-1* mutant strains predominantly advanced to the adult stage. Statistical analysis demonstrated significance for a difference of the wild-type and *pcm-1* mutants treated with 0.2 mM paraquat at the L2 larval stage (Supplemental Table S3). No significant statistical difference was observed between the two *pcm-1* mutants, nor was there a significant statistical difference between each *pcm-1* mutant and the wild-type animals following treatment with paraquat. We also subjected the *tm2679 C10F4.3* mutant to paraquat stress. We found no differences between this mutant and the wild type nematodes (Supplemental Figure S1; Table S4).

Since PCM-1 overexpression in the *PL51* strain enhances adult nematode lifespan at 25 °C (Banfield et al., 2008), we then asked whether there would be enhanced oxidative stress resistance in these animals. We thus repeated the experiments described above with the *PL51* strain overexpressing PCM-1, and the mutated *PL54* strain, overexpressing a catalytic

mutant form of PCM-1. Although we observed a similar developmental delay in the *PL54* strain and the *pcm-1* mutant animals, we found no difference between the PCM-1-overexpressing *PL51* strain and the wild-type N2 strain. However, we noted fewer lower stage larvae (L1 and L2) and young and gravid adults in the PCM-1 overexpressing rescue strain than in the wild-type strain following treatment with 0.2 mM paraquat (Supplemental Figure S2; Table S5).

To determine if the developmental delay was due to oxidative stress, wild-type and *pcm-1* mutant worm strains were incubated on plates containing both paraquat and vitamin C. Upon addition of either 0.5 or 1 mM vitamin C, the delay in development caused by paraquat was prevented in both wild-type and *pcm-1* mutant nematodes, and development into young and egg-laying adults occurred normally (Fig. 3, panel C).

3.3. Homocysteine and homocysteine thiolactone induce a delay in development that is more severe in *pcm-1* mutants

Homocysteine is a metabolite that has been linked to degenerative cardiovascular and neuronal pathologies (Jakubowski, 2007). The detrimental physiological effects associated with homocysteine may be mediated by reactive oxygen species that are produced following its auto-oxidation (Starkebaum 1986; Perna et al., 2003). Additionally, homocysteine can affect methylation reactions by reacting with adenosine to form *S*-adenosylhomocysteine, a potent inhibitor of many methyltransferases, including the L-isoaspartyl protein repair enzyme (Clarke and Banfield, 2001; Perna et al., 2003). A cyclized derivative of homocysteine, homocysteine thiolactone, is formed following intramolecular nucleophilic attack of its thiol group on its carboxylate group and can be hydrolyzed to give homocysteine. In the thiolactone form, homocysteine can covalently modify lysine residues within proteins and subsequently alter tertiary structure and induce protein aggregation (Jakubowski, 2007). Since homocysteine and homocysteine thiolactone can both induce oxidative stress and potentially inhibit protein repair reactions, we were interested in comparing the effects of these two compounds to those induced by the oxidizing agents juglone and paraquat on wild-type and *pcm-1* mutant nematodes. As a control, we utilized the inactive disulfide homocysteine.

Wild-type (N2) and *pcm-1* mutant (*qa201* and *tm363*) strains were exposed to homocysteine, homocysteine thiolactone and homocystine. In the lifespan analysis, adult nematodes (both wild-type and *pcm-1* mutant animals) treated with 10 mM or 20 mM of each of these compounds had very similar survival rates when compared to nematodes in untreated conditions (data not shown). However, in terms of development, homocysteine and homocysteine thiolactone caused delays in wild-type and *pcm-1* mutant nematodes that were more severe in the mutants. The delay in development in wild-type nematodes treated with homocysteine and homocysteine thiolactone was similar to the paraquat-induced developmental delay at the lower dosage (10 mM) but that were more severe at the higher dosage (20 mM) of homocysteine derivatives used (Fig. 4, panel A). Under the latter conditions, L2 larvae were the predominant stage present. In contrast, nematodes exposed to homocystine did not undergo a delay in development.

Pcm-1 mutant *C. elegans* (both *qa201* and *tm363* strains) underwent a more pronounced delay in development at the first larval stage (L1) in comparison to wild-type *C. elegans* following treatment with homocysteine and homocysteine thiolactone (Fig. 4, panel B; Supplemental Table S6). The percentage of wild-type *C. elegans* that delay at the L1 stage in 10 mM and 20 mM homocysteine is 1.6% and 6.2%, respectively, whereas the *pcm-1* mutants delay in L1 at 5% and 41%, respectively, under these conditions. The difference in the animals treated with 20 mM homocysteine is statistically significant (Fig. 4, panel B; Supplemental Table S6). Similar effects were seen following treatment with homocysteine thiolactone (Fig. 4, panel B).

Whereas a majority of the *pcm-1* mutant animals delay at the L1 stage following treatment with 20 mM homocysteine or homocysteine thiolactone (41% and 46%, respectively), approximately 50% of the wild-type animals delay at the second larval stage (L2) following treatment with these compounds (Fig. 4, panels A and B). We also showed that 20 mM homocysteine causes a statistically significant increase in the number of *pcm-1* mutant nematodes delayed in L2 as well, though relatively fewer than are seen for the wild-type nematodes (Fig. 4, panels A and B; Supplemental Table S6).

Statistical analysis was performed to further distinguish the effects of homocysteine on wild-type and *pcm-1* mutant development. We combined the data from a “control” group consisting of untreated animals and animals incubated with 10 mM and 20 mM of the inactive homocysteine derivative and an “experimental” group consisting of animals treated with 20 mM homocysteine and 10 mM and 20 mM homocysteine thiolactone. We found that the delay in development at the first and second larval stages in the experimental data set for *pcm-1* mutant animals was statistically significant (Supplemental Table S6).

As the detrimental effects of homocysteine can be mediated by oxidative stress, we then tested whether vitamin C could prevent the delay in development. The effects of 1 mM vitamin C on the homocysteine thiolactone-induced delay in development are shown in Fig. 5. In all cases, the developmental delay was prevented in the presence of vitamin C in both wild-type and *pcm-1* mutant animals. For animals treated with 10 mM homocysteine thiolactone and vitamin C, development proceeded normally to the adult stage for both the wild-type and *pcm-1* mutants. For animals treated with 20 mM homocysteine thiolactone and vitamin C, there were a small number of wild-type L4 larvae (about 10%) and *pcm-1* mutant L3 (12%) and L4 larvae (20%) remaining, indicating that the reversal was not complete.

Finally, we found no difference in development between wild-type and *tm2679 C10F4.3* mutant nematodes treated with homocysteine. When we tested the PCM-1 overexpression and mutant strains, *PL51* and *PL54*, we found similar results as with wild-type and *pcm-1* mutant animals, respectively, confirming the rescue of the phenotype by active PCM-1 enzyme (Supplemental Figure S2; Table S5).

3.4. Homocysteine treatment results in defects in egg laying (Egl phenotype)

Interestingly, not only did homocysteine induce a delay in development in *pcm-1* mutant animals, it also had detrimental effects on their ability to lay eggs as adults. This egg-laying defect was only present in the *pcm-1* mutant strain (Table 3). Following treatment with 20 mM homocysteine, approximately 75% and 42% of *pcm-1* mutant *qa201* and *tm363* animals displayed egg-laying defects, respectively. The addition of vitamin C (both 0.5 mM and 1 mM vitamin C) prevented the formation of the Egl defect in *pcm-1* mutant nematodes in a similar fashion to the juglone-treated Egl animals. Significantly, we did not observe the Egl phenotype in homocysteine thiolactone-treated nematodes.

4. Discussion

In mammals, oxidative stress can increase the cellular content of proteins containing age-damaged aspartyl residues that can be recognized for repair by the protein L-isoaspartyl methyltransferase (O'Connor et al., 1988; Ingrosso et al., 2000; Ingrosso *et al.*, 2002; D'Angelo et al., 2005; Zhu et al., 2006). However, the connection between oxidative and isomerization damage is not clear. This enzyme is widely conserved in nature, and we were thus interested in studying how *C. elegans*, a model organism for aging studies, responds to environmental oxidative stress conditions in the absence of the protein repair methyltransferase.

Upon exposure to the oxidizing agent juglone, we found that *pcm-1* mutant dauer larvae develop a defect in egg laying upon exiting dauer diapause. Previous research has shown juglone to be a potent oxidizing agent that is taken up directly by *C. elegans* (Blum and Fridovich, 1983; De Castro et al., 2004). Wild-type dauer larvae were able to combat the oxidative stress to exit dauer diapause, but *pcm-1* mutant dauer larvae were not, suggesting a defect in the neurosensory machinery necessary for normal vulval development and for the timely release of eggs. PCM-1 is expressed in the neural cords and neuronal cells, body wall, and reproductive tissues including the vulva (Gomez et al., 2008). Thus, the defect in egg laying observed in the juglone-treated *pcm-1* mutants may be due to damage in the vulva and/or neurons involved in signaling needed for egg-laying. Additional work is needed to determine what role PCM-1 plays in preventing defects in egg-laying imposed by stress, whether it be one involving protein repair or signaling.

The redox-cycling agent paraquat induced delays in development in wild-type and *pcm-1* mutant nematodes, but in the mutants the delay in development was more pronounced at the second larval stage. In comparison to juglone, paraquat exposure was much less toxic to wild-type and *pcm-1* mutant nematode larvae. It is unclear whether this distinction results from differences in the uptake or differences in the metabolism of both compounds by the nematodes. For example, paraquat is less permeable to *C. elegans* than juglone (Blum and Fridovich, 1983); it is possible that it is largely taken up by ingesting the paraquat-incubated *E. coli* food source. However, with both juglone and paraquat, defects were seen in the *pcm-1* mutant nematodes that were antagonized by vitamin C treatment, suggesting that elimination of reactive oxygen species prevents the effect of both of these drugs.

Significantly, *pcm-1* mutant nematodes treated with homocysteine displayed both Egl and developmental delay phenotypes, whereas the homocysteine thiolactone-treated *pcm-1* mutant nematodes experienced a delay in development only; both phenotypes induced by the homocysteine derivatives were prevented upon exposure to vitamin C. There is no information to our knowledge of how homocysteine and its derivatives are absorbed by *C. elegans*. Taken together, however, the results from the treatment of nematodes with juglone, paraquat, homocysteine and homocysteine thiolactone indicate that the loss of the PCM-1 repair methyltransferase significantly reduces the resistance of *C. elegans* to oxidative stress.

The role of vitamin C itself in the *C. elegans* oxidative stress response has not been extensively characterized. Initial examination of the effects of vitamin C has shown that, unlike vitamin E, it does not extend lifespan in *C. elegans* (Harrington and Harley, 1988). In fact, it is unclear whether vitamin C is synthesized in *C. elegans* or not. In our experiments, vitamin C treatment alone did not alter morphology or have any detrimental effects on development. However, our results clearly show that exogenous vitamin C can prevent the oxidative stress phenotypes in nematodes induced by juglone, paraquat, homocysteine, and homocysteine thiolactone; whether there are endogenous compounds that function in similar ways is not known.

As the effects of oxidative stress are either more pronounced (developmental delay) or are only observed (Egl phenotype) in *pcm-1* mutants, we hypothesize that PCM-1 may play an important role in the mechanisms by which *C. elegans* regulates stress response and development. It is possible that this role simply involves the repair of aspartyl isomerization damage in proteins and enzymes that are needed to respond to oxidative stress. However, it is also possible that the methyltransferase functions in a signaling role to up-regulate the expression of proteins that protect against oxidative stress. In mice, the loss of the PCMT1 gene for the repair methyltransferase has been shown to activate the insulin/insulin-like growth factor I pathway (Farrar et al., 2005). The mechanism of this activation is not known; it is possibly a cellular response to the increased concentration of damaged proteins. If a similar activation of the corresponding DAF-2 mediated insulin-like signaling pathway occurs in the *C. elegans*

pcm-1 mutants, the inactivation of the DAF-16 transcription factor would be expected to reduce the levels of superoxide dismutase, catalase, and HSP-70 and HSP-16 (McElwee et al., 2003). This effect may account in itself for the phenotypes observed in this work, or may contribute to it. Importantly, we have shown here that mutations in the *daf-2* insulin-like receptor gene effectively suppress the Egl phenotype of *pcm-1* mutant animals exposed to juglone. This result suggests that PCM-1 may be functioning as an upstream regulator of the DAF-2 signaling pathway, possibly by a similar mechanism to the one postulated for the control of dauer formation and survival by PCM-1 (Banfield et al., 2008). At this point, however, we cannot rule out that PCM-1 may give resistance to oxidative stress by a pathway parallel to the DAF-2 signaling pathway.

The interaction of PCM-1 and the DAF-2 pathway may also explain the more pronounced delay in development seen in *pcm-1* mutants under oxidative stress. The possible up-regulation of DAF-2 signaling leading to DAF-16 deactivation in *pcm-1* mutants could account for the inhibition of G2/M progression that would delay normal progression through the L1 to L4 larval stages (Furukawa-Hibi et al., 2005; Wolf et al., 2007). Furthermore, it has been demonstrated that a mutant in the DAF-2 signaling pathway displays similar characteristics to the *pcm-1* mutant animals exposed to juglone or homocysteine. Specifically, *C. elegans* lacking the serum- and glucocorticoid-inducible kinase 1 (SGK-1), a protein that forms a complex with protein kinase B of the DAF-2 pathway, undergoes a delay in development and displays the Egl phenotype (Hertweck et al., 2004). Further work will be needed to determine the mechanism(s) by which PCM-1 plays a beneficial role in oxidative stress resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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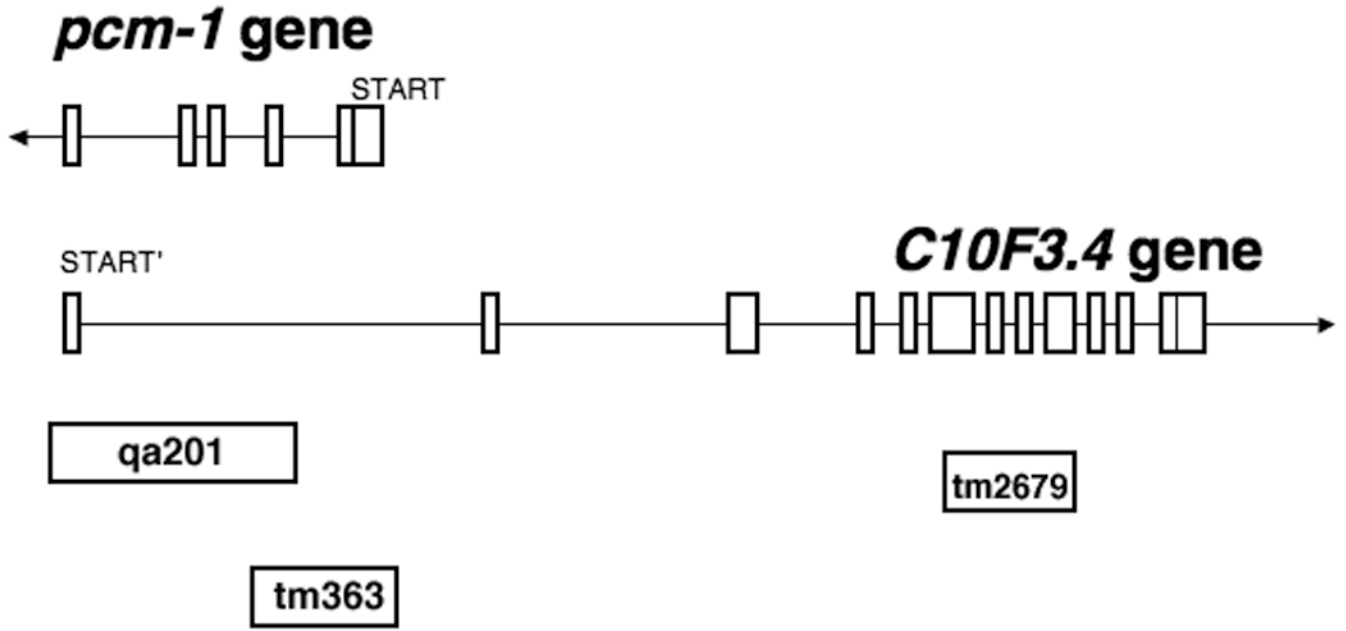
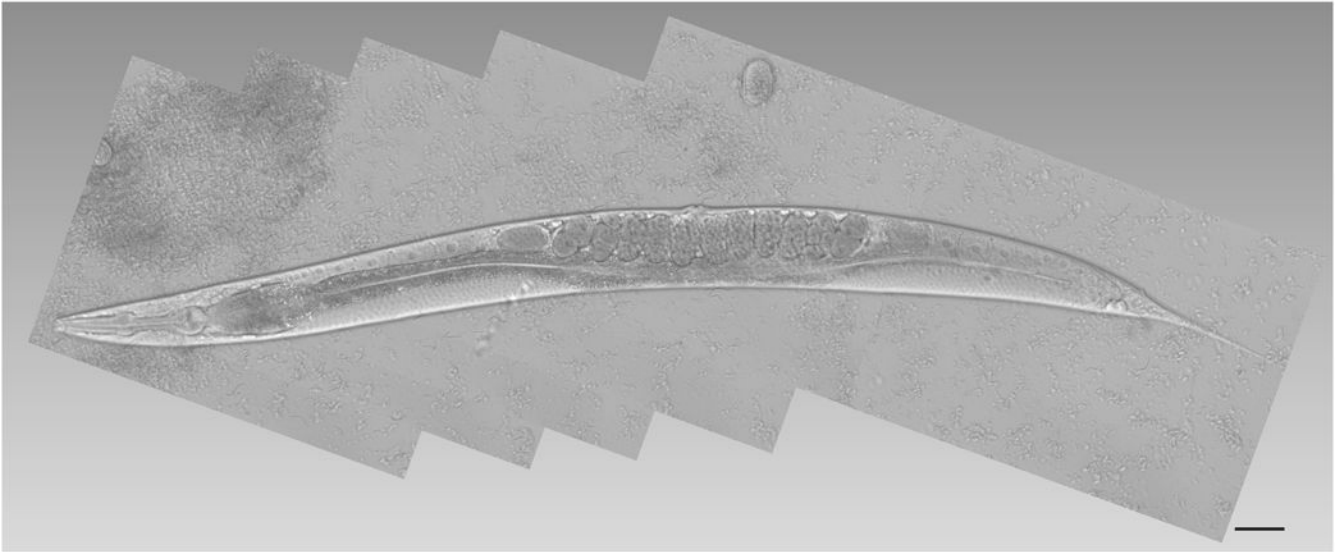
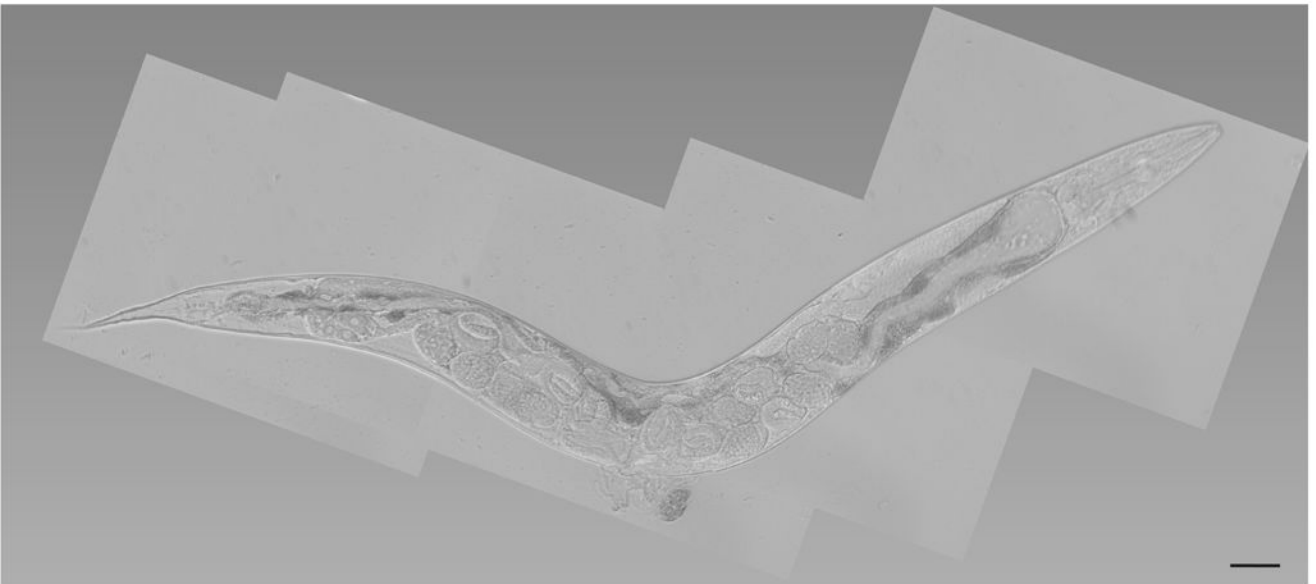


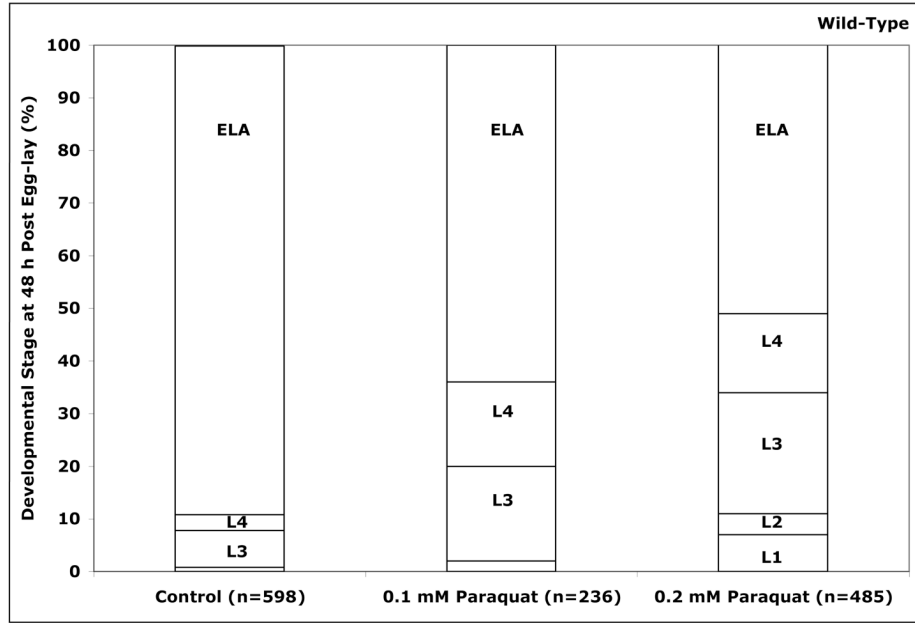
Fig. 1. Genetic organization of the *C. elegans* *PCM-1* region
Gene map for *C. elegans pcm-1* and the partially overlapping *C10F3.4* gene. Exons are shown by boxes; introns by lines. The regions deleted in each mutant are indicated; the direction of transcription is shown with an arrow at the end of the transcript.

pcm-1 (qa201): control***pcm-1 (qa201): juglone*****Fig. 2.**

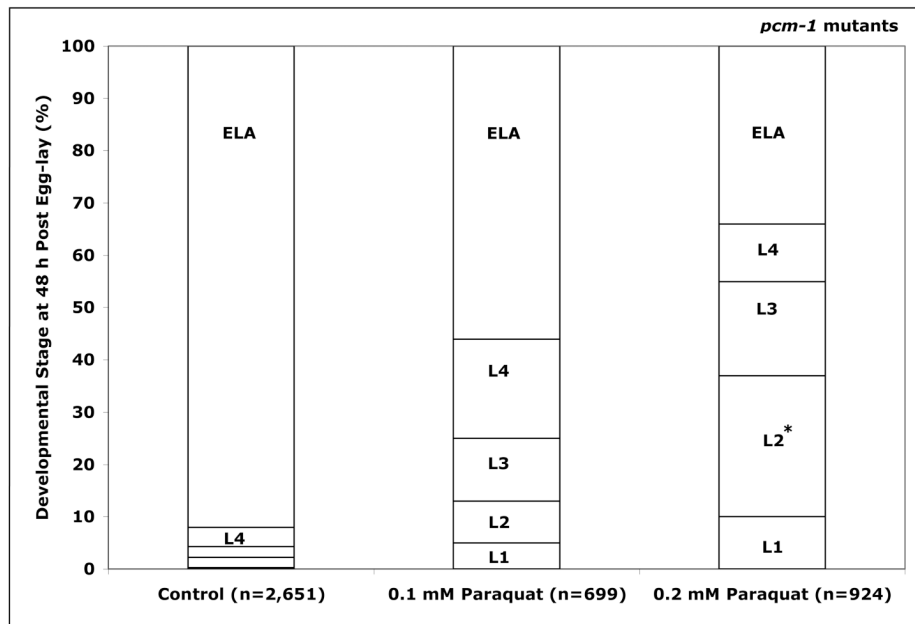
Juglone-treated *pcm-1(qa201)* mutant dauer larvae develop a defect in egg-laying as gravid adults. *Pcm-1(qa201)* mutant dauer larvae (total of 300 animals) were incubated in liquid droplets containing either S-media (control) or S-media with 236 μ M juglone for 90 min and were transferred NGM + OP50 plates. The animals were monitored for development and viability every 24 hr for 3 days. Most of the 150 control *qa201* mutant dauers developed into healthy gravid adults as shown by Nomarski differential interference contrast microscopy at 200-fold magnification (upper panel), with some animals only progressing to L3 or L4 larvae. On the other hand, about one fifth of the 150 juglone-treated mutant dauer larvae developed

into adults with a defect in egg-laying shown in the lower panel, with the remainder progressing apparently normally to L3, L4, and gravid adults. Scale bar = 50 μ m.

A.



B.



C.

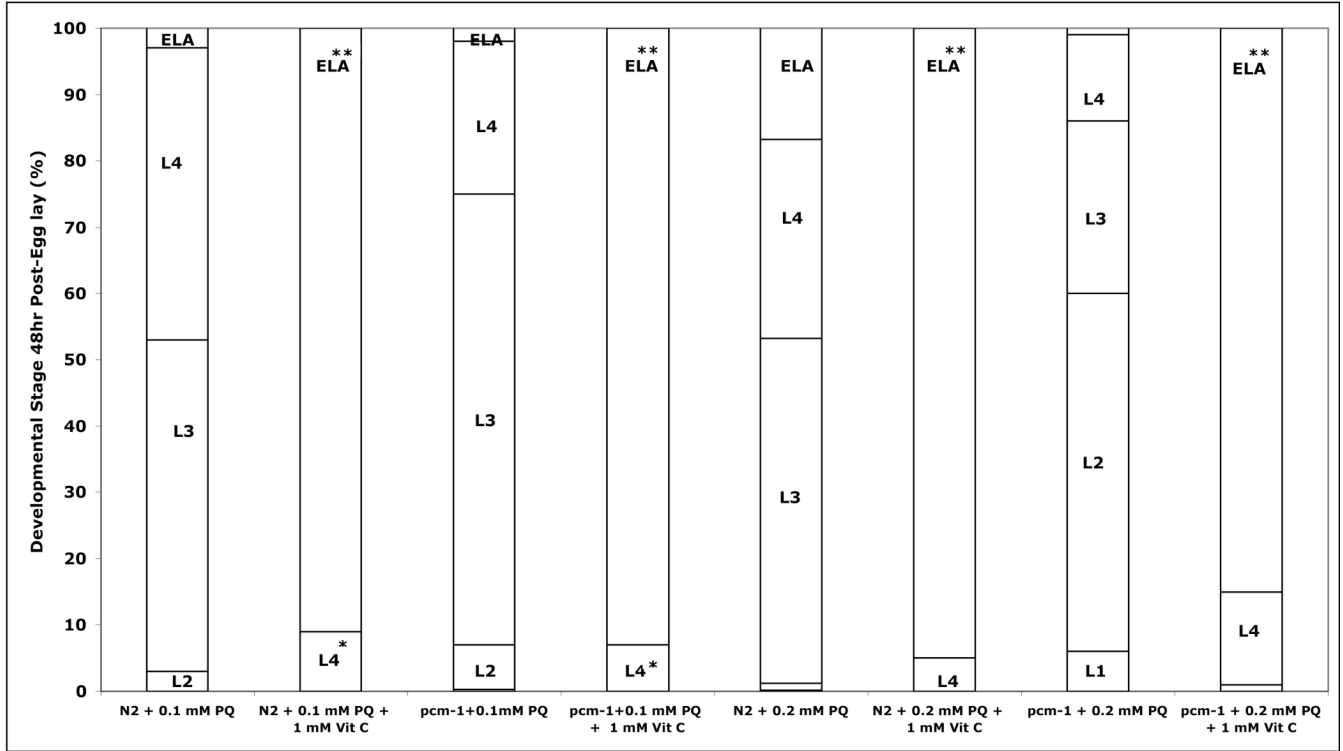


Fig. 3.

(A) Paraquat delays development in wild-type (N2) *C. elegans*. Eggs (about 100) were transferred to each of two NGM+OP50 plates containing 0, 0.1 mM and 0.2 mM paraquat at 25 °C. The developmental stage of each worm was determined after 48 h as L1, L2, L3, and L4 larvae and as young adult/egg-laying adult (YA/ELA) nematodes. Data from four replicate experiments (2 plates each) are averaged. Asterisks indicate a statistically significant difference in developmental delay between control and treated animals (two-tailed Student’s t-test of unequal variance: * $p < 0.05$, ** $p < 0.005$). (B) Paraquat treatment results in more severe developmental delays in *pcm-1* mutant nematodes. Eggs from *qa201* (*pcm-1* mutant) and *tm363* (*pcm-1* mutant) were incubated on paraquat-containing plates and the developmental stage was identified as above. Data from each mutant set were combined. (C) Vitamin C reverses the delay in development induced by paraquat in *C. elegans*. Eggs from three *C. elegans* strains (N2 (wild-type), *qa201* (*pcm-1* mutant), and *tm363* (*pcm-1* mutant)) were transferred to NGM+OP50 plates containing 0, 0.1 mM and 0.2 mM paraquat in the presence and absence of 1 mM vitamin C and were then scored for larval development after 48 h as above. Data from four replicate experiments are shown; the data from the two mutant strains were pooled. Asterisks indicate a statistically significant difference in developmental delay between wild-type and *pcm-1* mutant animals treated under the paraquat versus paraquat plus vitamin C concentrations (two-tailed Student’s t-test of unequal variance: * $p < 0.05$, ** $p < 0.005$). The numbers of animals scored in each of the groups in this figure are given in Supplemental Table S7 along with the standard deviation values for the percentage of each larval stage under each condition.

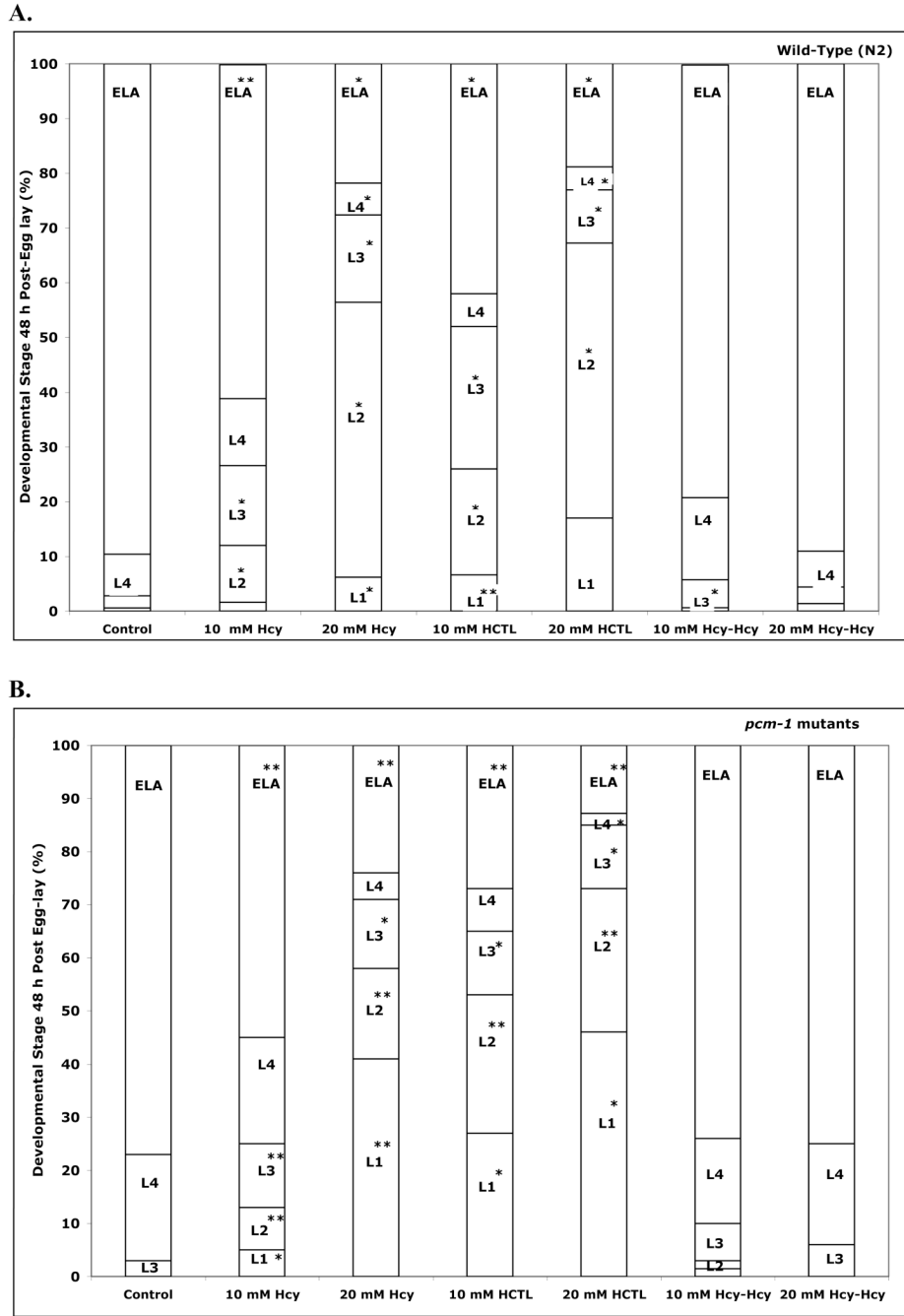


Fig. 4. (A) Homocysteine and homocysteine thiolactone delay development in wild-type (N2) *C. elegans*. Eggs (about 100) were transferred to NGM+OP50 plates containing 0, 10 mM and 20 mM homocysteine (Hcy), homocysteine thiolactone (HCTL), and homocysteine (Hcy-Hcy) and the developmental stage of each worm was determined after 48 h at 25 °C as L1, L2, L3, and L4 larvae and as young adult/egg-laying adult (YA/ELA) nematodes. Data from a total of five experiments each with duplicate plates are shown. Asterisks indicate a statistically significant difference in developmental delay between control and treated animals (two-tailed Student’s t-test of unequal variance: *p < 0.05, **p < 0.005). (B) Homocysteine treatment results in more severe developmental delays in *pcm-1* mutant nematodes. Eggs from *qa201*

and *tm363 pcm-1* mutants were exposed to homocysteine, homocysteine thiolactone, and homocystine and development was scored as above, again with five duplicate experiments for each mutant. Asterisks indicate a statistically significant difference in developmental delay between wild-type and *pcm-1* mutant animals treated under the specified homocysteine compound concentrations (two-tailed Student's t-test of unequal variance: * $p < 0.05$, ** $p < 0.005$). The numbers of animals scored in each of the groups in this figure are given in Supplemental Table S7 along with the standard deviation values for the percentage of each larval stage under each condition.

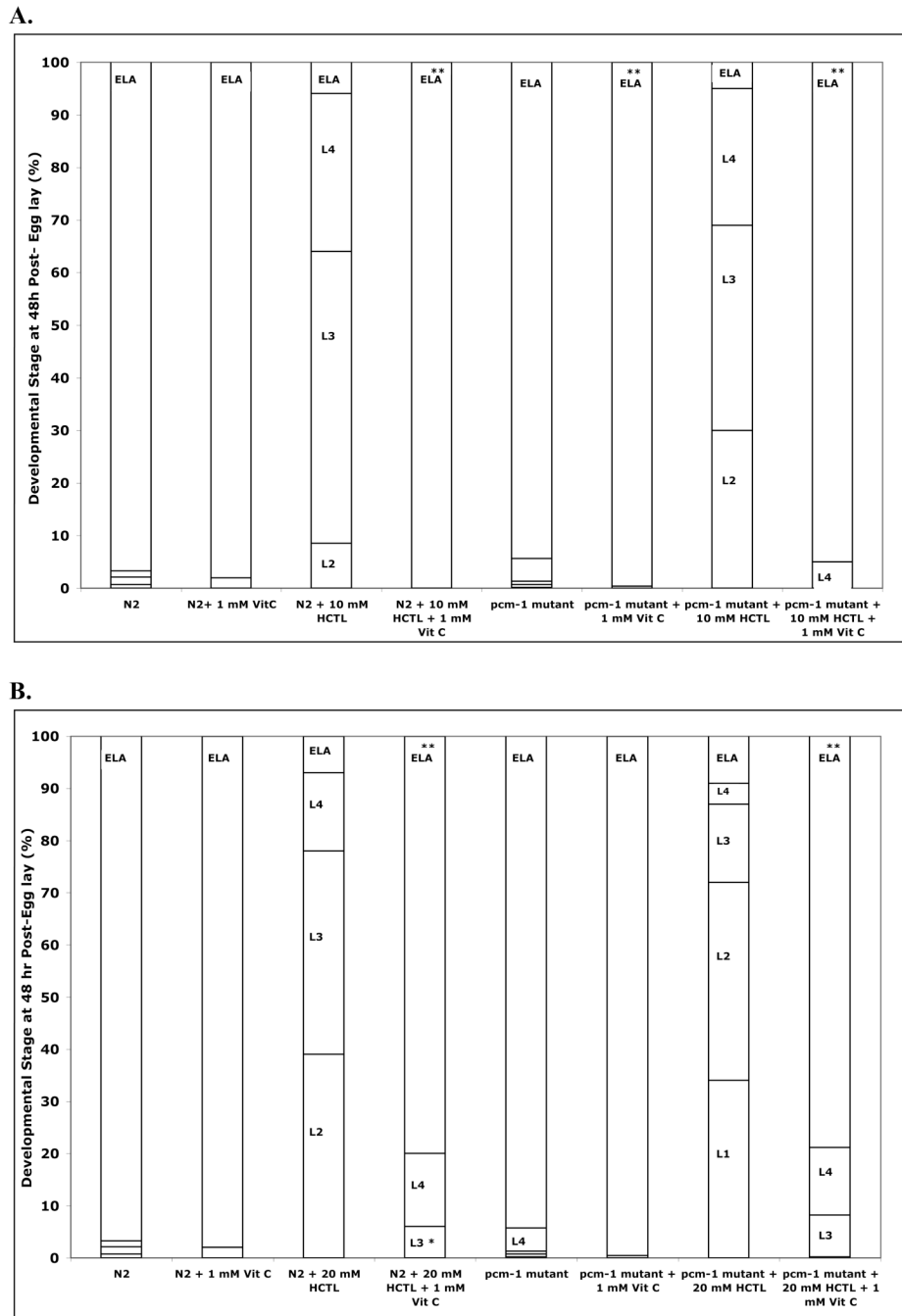


Fig. 5. Vitamin C reverses the delay in development induced by homocysteine thiolactone (HCTL) in *C. elegans*. Eggs (about 100) from strains N2 (wild-type), *qa201* (*pcm-1* mutant), and *tm363* (*pcm-1* mutant) were transferred to duplicate NGM+OP50 plates containing 0 and 10 mM homocysteine thiolactone (*panel A*) or 0 and 20 mM homocysteine thiolactone (*panel B*) in the presence and absence of 1 mM vitamin C at 25 °C and scored for larval development at 48 h as in Fig. 4. Data are shown for four replicate experiments. Data for the two *pcm-1* mutants are combined. Asterisks indicate a statistically significant difference in developmental delay between wild-type and *pcm-1* mutant animals treated under the specified homocysteine compound versus homocysteine plus vitamin C concentrations (two-tailed Student’s t-test of

unequal variance: * $p < 0.05$, ** $p < 0.005$). The numbers of animals scored in each of the groups in this figure are given in Supplemental Table S7 along with the standard deviation values for the percentage of each larval stage under each condition.

Juglone-treated L-isoaspartyl protein methyltransferase-deficient dauer larvae develop a defect in egg laying (Egl) upon refeeding which is blocked by the addition of vitamin C. Dauer larvae were incubated in the presence or absence of 236 μ M juglone with or without 1 mM vitamin C and analyzed for the Egl phenotype as described in "Experimental Procedures". Three replicate experiments were completed with the total number of animals given (n). The p-value for the difference between the juglone-treated and untreated larvae is given from the two-tailed Student's t-test of unequal variance.

Table 1

Strain	n	Juglone		Vitamin C		% Egl phenotype	p-value
		-	+	-	+		
N2	89	-	-	-	-	0	
	169	-	-	+	+	0	
	140	+	+	-	-	0	
	150	+	+	+	+	0	
	71	-	-	-	-	0	
<i>pcm-1(qa201)</i>	150	-	-	+	+	0	
	142	+	+	-	-	28 \pm 3.0	2.1 \times 10 ⁻⁵
	146	+	+	+	+	0	
	75	-	-	-	-	0	
<i>pcm-1(tm363)</i>	90	-	-	+	+	0	
	84	+	+	-	-	24 \pm 4.0	1.6 \times 10 ⁻⁴
	100	+	+	+	+	0	
	60	-	-	-	-	0	
<i>C10F3.4(mm2679)</i>	45	-	-	+	+	0	
	52	+	+	-	-	5 \pm 2.3	0.74
	68	+	+	+	+	0	

Table 2

An additional mutation in the *daf-2* gene of the insulin-like signaling pathway suppresses the Egl phenotype displayed by *pcm-1* mutant dauer larvae following exposure to juglone. Dauer larvae were treated with juglone at 236 μ M and/or vitamin C at 1 mM as described in Table 1 and the percentage of animals displaying a defect in egg laying (Egl) is shown at 24 h, along with the percent animals that are dauer and L3 larvae at 48 h. The results are shown from three to six replicate experiments \pm standard deviation, with the number of animals scored (n). Each experiment utilized 40–50 dauer larvae from each strain.

Strain	n	Juglone	Vitamin C	% Egl phenotype	% dauer	% L3
N2	140	-	-	0	0	0
	133	-	+	0	0	0
	145	+	-	0	0	0
	130	+	+	0	0	0
<i>daf-2(m41)</i>	270	-	-	0	0	0
	304	-	+	0	0	0
	321	+	-	0	0	0
	282	+	+	0	0	0
<i>pcm-1(qa201)</i>	150	-	-	0	0	2.0 \pm 2.0
	150	+	-	21.3 \pm 2.3	0	0
<i>daf-2(e1370);pcm-1(qa201)</i>	438	-	-	0	0	3.0 \pm 4.1
	323	-	+	0	0	0
	472	+	-	0	0	1.8 \pm 3.4
	323	+	+	0	0	0
<i>daf-2(m596);pcm-1(qa201)</i>	299	-	-	0	0	0
	299	-	+	0	0	0
	316	+	-	0	1.5 \pm 2.5	0
	324	+	+	0	0	1.5 \pm 2.1

Homocysteine-treated L-isoaspartyl protein methyltransferase-deficient eggs develop into gravid adults exhibiting a defect in egg laying (Egl phenotype) which is prevented in the presence of vitamin C. Eggs (about 100) were transferred to NGM+OP50 plates containing 0, 10 mM and 20 mM homocysteine (Hcy) in the absence or presence of 1 mM vitamin C. The developmental stage of each worm was determined after 48 h at 25 °C as L1, L2, L3, and L4 larvae and as young adult/egg-laying adult (YA/ELA) nematodes. The percentage of ELAs displaying a defect in egg laying (Egl phenotype) was determined. Data from a total of five experiments each with duplicate plates are shown, with the number of animals given (n). Statistical analyses show that the differences in Egl phenotype between the wild-type and *pcm-1* mutant *C. elegans* worm strains were significant following exposure to 20 mM homocysteine.

Table 3

Strain	n	20 mM Homocysteine	1 mM Vitamin C	% Egl phenotype	p-value
N2	307	-	-	0	
	435	-	+	0	
	73	+	-	0	
	435	+	+	0	
<i>pcm-1(qa201)</i>	129	-	-	0	
	400	-	+	0	
	34	+	-	75 ± 25	1.1 × 10 ⁻⁵
	550	+	+	0	
<i>pcm-1(tm363)</i>	100	-	-	0	
	439	-	+	0	
	41	+	-	42 ± 16	1.6 × 10 ⁻⁵
	528	+	+	0	
<i>C10F3.4(tm2679)</i>	410	-	-	0	
	392	-	+	0	
	40	+	-	0	
	82	+	+	0	