The Interplay between Protein L-Isoaspartyl Methyltransferase Activity and Insulin-Like Signaling to Extend Lifespan in Caenorhabditis elegans

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Abstract

The protein L-isoaaspartyl-O-methyltransferase functions to initiate the repair of isomerized aspartyl and asparaginyl residues that spontaneously accumulate with age in a variety of organisms. Caenorhabditis elegans nematodes lacking the pcm-1 gene encoding this enzyme display a normal lifespan and phenotype under standard laboratory growth conditions. However, significant defects in development, egg laying, dauer survival, and autophagy have been observed in pcm-1 mutant nematodes when deprived of food and when exposed to oxidative stress. Interestingly, overexpression of this repair enzyme in both Drosophila and C. elegans extends adult lifespan under thermal stress. In this work, we show the involvement of the insulin/insulin-like growth factor-1 signaling (IIS) pathway in PCM-1-dependent lifespan extension in C. elegans. We demonstrate that reducing the levels of the DAF-16 downstream transcriptional effector of the IIS pathway by RNA interference reduces the lifespan extension resulting from PCM-1 overexpression. Using quantitative real-time PCR analysis, we show the up-regulation of DAF-16-dependent stress response genes in the PCM-1 overexpressor animals compared to wild-type and pcm-1 mutant nematodes under mild thermal stress conditions. Additionally, similar to other long-lived C. elegans mutants in the IIS pathway, including daf-2 and age-1 mutants, PCM-1 overexpressor adult animals display increased resistance to severe thermal stress, whereas pcm-1 mutant animals survive less long under these conditions. Although we observe a higher accumulation of damaged proteins in pcm-1 mutant nematodes, the basal level of isoaspartyl residues detected in wild-type animals was not reduced by PCM-1 overexpression. Our results support a signaling role for the protein L-isoaaspartyl methyltransferase in lifespan extension that involves the IIS pathway, but that may be independent of its function in overall protein repair.

Introduction

A master regulator of aging in the nematode Caenorhabditis elegans is the insulin/insulin-like growth factor-1 signaling (IIS) pathway. The IIS pathway is conserved in other organisms, including Drosophila melanogaster and mammals [1]. Activation of the DAF-2 insulin-like receptor in C. elegans triggers the successive phosphorylation and activation of several kinases including AGE-1 (Class IA phosphatidylinositol-3-kinase) and AKT (protein kinase B); the activated AKT kinase can subsequently phosphorylate the FoxO transcription factor DAF-16, excluding it from the nucleus and thereby rendering it inactive [2,3]. However, when this kinase cascade of phosphorylation events is deactivated, the DAF-16 transcription factor enters the nucleus, leading to the enhanced expression of genes involved in longevity, dauer formation, and stress resistance [2,3]. C. elegans mutants lacking a functional insulin-like receptor or PI3-kinase (daf-2 and age-1 mutants, respectively), display lifespan extensions spanning two-fold to ten-fold and show enhanced stress resistance [4–6]. The long-lived phenotype for the daf-2 pathway mutants is abolished upon additional mutation of the DAF-16 transcription factor [5,7,8].

Another protein implicated in C. elegans longevity is the protein L-isoaaspartyl [D-aspartyl]-O-methyltransferase (PCM-1), the product of the pcm-1 gene [9]. Known primarily for its protein repair role in higher organisms, this highly conserved enzyme specifically recognizes damaged aspartyl and asparaginyl residues that accumulate with age [10]. Following the spontaneous deamination and/or isomerization of these residues, protein backbones become kinked. The repair enzyme methylates the α-carboxyl group of isoaspartyl residues, thereby facilitating “unkinking” of the backbone and conversion of the isomerized residues back to normal L-aspartyl residues [10]. E. coli lacking the isoaspartyl methyltransferase gene display increased susceptibility to various environmental stresses [11]. Knockout mice in the corresponding Pent1 gene accumulate altered proteins, show growth retardation, and are subject to premature death from fatal seizures at an average age of 42 days [12]. In plants, absence of the repair methyltransferase has been shown to be involved in decreased seed longevity.
and germination vigor in Arabidopsis thaliana [13]. On the contrary, overexpression of the methyltransferase in E. coli results in increased survival under heat shock [14] and oxidative stress [15] and extended lifespan under thermal stress in D. melanogaster [16] and C. elegans [17].

Further characterization of this repair enzyme has revealed interplay between PCMT and major intracellular signaling pathways, including the IIS pathway and the mitogen-activated protein kinase (MAPK) pathway. For example, *Pom1* knockout mice showed activation of the IIS signaling pathway as evidenced by increased phosphorylation of kinases including AKT, glyco-gen synthase kinase-3 beta (GSK-3β), and PKD-1 [18]. In relation to the MAPK pathway, PCMT1-knockdown human cells stimulated with EGF (epidermal growth factor) showed increased phosphorylation of MAPK pathway components, including Raf-1, MEK, and ERK1/2 [19,20]. Additionally, Doyle and colleagues found that the absence of PCMT1 in mammalian lymph node cells causes T-cell hyperproliferation and increased phosphorylation of MAPK pathway components MEK1/2, ERK1/2, and RSK1 in response to CD3 receptor stimulation [21]. In contrast, enhanced PCMT1 expression induced by both valproic acid and lithium was accompanied by inactivation of GSK-3β in astrocytoma and neuroblastoma cells [22,23]. These findings illustrate that, in addition to protein repair, PCMT1 might be directly or indirectly involved in the regulation of the IIS pathway and/or the MAPK pathway in mammalian systems.

As opposed to mice, effects of a deficiency in the protein L-isoaspartyl-O-methyltransferase resulting from mutations in the *pcm-1* gene in *C. elegans* become apparent only under certain stress conditions. In control conditions, *pcm-1* mutants display a similar lifespan and brood size when compared to wild-type (*N2*) animals [24]. Under starvation conditions, *pcm-1* mutants display defects in dauer formation and survival as well as L1 larval survival [17,25]. During dauer formation, *pcm-1* mutants display reduced levels of autophagy, indicative of enhanced insulin/TOR signaling in the absence of PCMT1 [25]. Furthermore, under oxidative stress conditions, we have recently shown that *pcm-1* mutants display defects in egg laying as well as delayed larval development [26]. An additional mutation in the *daf-2* gene in the *pcm-1* mutants restored a normal dauer lifespan [17] and recovered stress defects in larval development and egg laying [26], indicating an epistatic relationship between genes of the IIS pathway and the gene encoding the isoaspartyl methyltransferase.

Given the normal adult lifespan of *pcm-1* mutant *C. elegans*, it was a surprise to find that PCM-1 overexpression extends adult lifespan two-fold compared to wild-type nematodes under mild thermal stress [17]. As it was previously shown that the lifespan extension of long-lived IIS pathway mutants is DAF-16-dependent, this work is focused on determining whether the prolonged lifespan of PCM-1 overexpressor animals also involves the transcriptional activity of DAF-16. Furthermore, the importance of the protein repair function of PCM-1 in this context has been addressed.

**Methods**

**Worm strains and procedures**

Standard procedures used to maintain *C. elegans* strains were adapted from Sulston and Hodgkin [27]. The *N2* strain was obtained from the Caenorhabditis Genetics Center (St. Paul, MN). Two transgenic strains (*PL51* and *PL54*) along with the *pcm-1* (qa201) mutant strain were used for all experimental procedures [17]. Strain *PL51* is a non-integrated strain that was created by injection of a plasmid expressing the *pcm-1* gene under its endogenous promoter into a *pcm-1* mutant background. Three to seven-fold higher isoaspartyl methyltransferase activities were measured in transgenic *PL51* worm extracts compared to wild-type *N2* extracts prepared from animals grown in liquid culture [26]. Strain *PL54* contains a similar plasmid (also in a *pcm-1* mutant background) to the *PL51* strain except that it encodes a PCM-1 protein with two mutated residues in the enzyme’s AdoMet cofactor binding site (*G90V* and *G90T*). Extracts prepared from *PL54* nematodes did not demonstrate any methyltransferase activity [26]. The *pcm-1* (qa201) mutant lacks exons 2–3 of the *pcm-1* gene [24] and alternative exon 1 from the partially overlapping *G88V*) strain [26]. This allele was backcrossed eight times into an N2 background [17] to obtain the *pcm-1* (qa201) strain, which also totally lacks methyltransferase activity. Protein L-isoaspartyl O-methyltransferase activity was measured again in each of the strains used in this study and values are provided in Table 1. The integrated DAF-16:GFP roller strain TJ356 was used to test *daf-16* RNAi efficacy and to monitor subcellular DAF-16 localization under various conditions. This strain was kindly given to us by Dr. Brian Head (UCLA Department of Biological Chemistry). In the absence of *daf-16* RNAi, we confirmed expression of the GFP-tagged DAF-16 protein in most cell types (including the hypodermis, neurons, intestine, and gonad) except the pharynx in the TJ356 strain [26]. With *daf-16* RNAi, we observed a loss of fluorescence in all the *daf-16* expressing regions except for neurons. The efficiency of *pcm-1* RNAi was tested with the *xEx104 (UZ122)* strain expressing a PCM-1::GFP fusion protein [25]. In the absence of *pcm-1* RNAi, we confirmed PCM-1 expression (body wall, reproductive tissues, and neurons) as initially reported by Gomez et al. [25] and observed a decrease in fluorescence (except in neurons) following *pcm-1* RNAi treatment in the UZ122 strain. All strains were kept at 20°C or 25°C on nematode growth medium plates streaked with *E. coli* OP50 (NGM + OP50) [27].

**Lifespan analysis and RNAi**

The protocol used to complete the lifespan studies was adapted from Sulphin and Karberlein [29]. First, eggs from three strains (*N2, PL51, and PL54*) were collected following hypochlorite treatment of gravid adults and were transferred to NGM + OP50 plates. Following an approximately 48 h incubation at 20°C, L4

| Table 1. PCM-1 activity measurements in various *C. elegans* strains. |
|---------------------------------------------------------------|
| **Strain** | **Methyl Groups Transferred (pmol/min/mg protein)** |
|----------|---------------------------------------------------|
| *N2*     | 0.60±0.04                                        |
| *pcm-1* (qa201) | 0.01±0.03                                 |
| *PL51*   | 1.97±0.26                                        |
| *PL54*   | −0.10±0.01                                       |

Nematode protein extracts were prepared from liquid culture and analyzed for PCM-1 methyltransferase activity as described by Kagan et al. (1997). Protein extracts were incubated with the methyl-accepting peptide substrate KASA-isoD-LAKY (100 μM) and 20 μM [14C]-AdoMet at 30 °C for 2 h. Control incubations were conducted as above except that KASA-isoD-LAKY was not added to the reaction. Protein L-isoaspartyl O-methyltransferase activity levels are given as the amount of methyl groups transferred after correction for the background levels measured in the absence of KASA-isoD-LAKY for the various *C. elegans* strains used in the lifespan analyses, toleromerase assays, RT-qPCR analyses, and isoaspartyl quantifications performed in this study. The results shown are means (± SD) of two separate activity measurements conducted with one set of lysates.

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larvae from each strain were transferred to NGM plates containing isopropyl-β-D-1-thiogalactopyranoside at 1 mM (pem-1 RNAi) or 2 mM (daf-16 RNAi) (IPTG; Anatrace, Inc. #I-1003), 0.025 mg/ml carbenicillin sodium salt (CB; Sigma #C-1389), and 45 μM 5-fluorodeoxyuridine (FUDR; Sigma #F6875) and streaked with RNAi-expressing bacteria (NGM + IPTG + CB + FUDR + RNAi). Following transfer (day 0), lifespan plates were incubated at 25°C until final scoring for survival was completed (survival was scored every other day until all animals were dead). Animals were considered dead when no signs of viability (motility, pharyngeal pumping, and response to platinum wire probing) were detectable. Lifespan experiments were repeated three times. Averages of mean and maximum survival were determined and examined for statistical significance using the ANOVA test for significance.

The RNAi bacteria (either scrambled control or daf-16 (1-5m24)) were obtained from the Ahringer library [30] kindly provided by Dr. Brian Head at the UCLA Department of Biological Chemistry. The pem-1 RNAi feeding clone, derived from the C. elegans ORFeome Library v1.1 [31], was obtained from Open Biosystems (Huntsville, AL). Several clones were derived from the original bacterial stock; the clones #2 and #3 used in this study are lacking exon 3 or part of exon 2 of the pem-1 gene. The identity of the RNAi clones was verified by sequencing using the sjj_R13H8.1 primers (listed on www.wormbase.org as sjj_R13H8.1-F: AGTACGCAAATTCC-CAAATGAAA and sjj_R13H8.1-b: ATTTGATTTCCGAG-fAAGTGGAT) for the daf-16 gene and the pL4440-dest-RNAi universal primers (For: GTTTTCGACCAGTCAAGCGTT, Rev: TGGATAACCGTATTACCGCC) for the pem-1 gene. For pem-1 RNAi, individual RNAi bacterial colonies were grown in LB + 50 μg/ml ampicillin for 7 h at 37°C; bacteria were then seeded onto NGM + IPTG + CB + FUDR plates and allowed to grow for 72 h before L4 larval transfer.

Thermotolerance assays

The protocol developed for this assay was adapted from Lithgow et al. [32], except that day 1 adult animals were tested at 37°C instead of 35°C. L4 larvae from each strain were transferred (20–30 animals per trial) to NGM + OP50 plates and were placed at 20°C overnight. The next morning, plates were transferred to 37°C and, at the indicated times, nematodes were scored for viability (motility, pharyngeal pumping, and response to platinum wire probing). Animals were considered dead when no signs of viability were detectable. The assay was repeated in triplicate and significance of survival time points was determined using the ANOVA test for significance.

Microscopy Assays

To monitor the subcellular localization of DAF-16 under various experimental conditions, the TJ356 strain was used. For the microscopy assays at 25°C, the progeny of RNAi-fed TJ356 L4 larvae were grown at 25°C until adulthood in both control and pem-1 RNAi conditions and were scored for DAF-16 localization as done previously by Henderson and Johnson [28]. For the microscopy assays at 37°C, the progeny of RNAi-fed TJ356 L4 larvae were grown in the same manner, except that animals were kept at 20°C. At day 1 of adulthood, the control RNAi-treated and pem-1 RNAi-treated nematodes were incubated at 37°C for two hours and DAF-16 subcellular localization was determined [28].

Reverse transcription and quantitative real-time PCR

RNA extraction and reverse transcription. For all four strains tested (N2, PL51, PL54, pem-1 (qa201)), gravid adults were treated for 3 min in a final concentration of 1.9 % sodium hypochlorite and 0.3 M NaOH. After five washes with M9 media, eggs were collected and starved overnight in M9 media to obtain synchronized L1 larvae. The L1 larvae were then transferred to NGM + OP50 plates and were incubated at 25°C for 48 h until nematodes had reached day 1 of adulthood. Day 1 adults were collected in M9 media (n = 200), triplicate samples for each strain) and stored at −80°C. For total RNA extraction, animals were homogenized in 1 ml TRI Reagent® (Molecular Research Center, #TR-118) with 1% β-mercaptoethanol (BioUltra, Sigma, #H3689) using a Polytron PT2000 homogenizer with a 7 mm generator. Phase separation of RNA from DNA and proteins was completed using bromochloropropane (Molecular Research Center, #BP-151). RNA was DNase-treated and further purified using the RNaseaEasy Fibrous Tissue Mini Kit (Qiagen; Cat #74704).

Following quantitation of RNA by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (the yield was generally about 4 μg RNA per 200 animals) and verification of absence of protein contamination by 260/280 nm ratio determination (between 1.8 and 2.0), a sample was run on a 1% TAE agarose gel to check for RNA degradation. RNA integrity was noted by observation of two sharp and discrete 26S and 18S ribosomal bands and the absence of leading smears, genomic DNA, and extra bands in each well. DNA-free RNA (2 μg in a total volume of 40 μl) was then converted to complementary DNA using oligo (dT) primers and a two-step RT-PCR procedure with heat denaturation (RETROscript Kit, Ambion #AM1710) according to the manufacturer’s instructions.

Quantitative real-time PCR and qPCR primers. Amplification reactions were carried out in a total volume of 20 μl containing the SYBR Premix Ex Taq reagent (Clontech Laboratories, Inc., Takara Product #TAK RR041A), 0.3 μM of gene-specific forward and reverse primers, and cDNA corresponding to 100 ng input RNA in the reverse transcriptase reaction. The reaction conditions for the Opticon 2 system (MJ Research) were: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s. The fluorescence was measured at each cycle at 80°C. Melting curves were performed after the PCR to assess the presence of a unique final product, and for each primer pair the product from one reaction was run on an agarose gel to further verify specificity and to confirm the expected band size.

The gene expression data are presented as the fold-change in mRNA transcription abundance in transgenic or mutant worm strains, normalized to two endogenous reference genes (cde-42 and ama-1), relative to the wild-type N2 strain. cde-42 encodes the RHO GTPase and ama-1 encodes RNA polymerase II; both genes were previously validated as relatively reliable reference genes to use for quantitative gene expression studies in C. elegans [33]. The expression results were similar when normalized against cde-42 or ama-1 individually. Normalized fold-changes (and statistical significance) were calculated using the REST 2009 software (Qiagen).

Quantitative real-time PCR primers were newly designed for the C. elegans genes ama-1 (forward primer: 5’-CGGTCGA- AAGGCTATCGAG-3’; reverse primer: 5’-CCAACCTCCTGCACGAT-3’), pem-1 (forward primer: 5’-TGTATGTGATCACGAT-3’; reverse primer: 5’-ACGACGCTCTTGACGAT-3’), mtl-1 (forward primer: 5’-GTTAAGCATG-3’; reverse primer: 5’-GAAAGAAGTGGTACGATT-3’). Previously published primer pairs were used for the C. elegans genes cde-42 [33], sod-3, daf-16 [34], mtl-1, dod-3, P21F3.3, dod-22, and dod-24 [35].

Quantification of protein substrates for the L-isospartyl methyltransferase in C. elegans extracts

Nematodes from each strain (N2, PL51, and pem-1 (qa201)) were grown at either 20°C or 25°C for 5 days in liquid cultures.
(S-media supplemented with concentrated E. coli OP50) and harvested by washes and sucrose flotation as previously described [24]. Preparation of C. elegans cytosol and determination of protein concentration therein was also done as previously [24]. To quantify the level of isoaspartyl methyltransferase substrates in the various protein extracts, base-labile methyl ester groups were assayed in those extracts by a method modified from that of Kagan et al. [24]. Specifically, nematode cytosolic extracts (125 μg protein) were incubated in a 50 μl reaction mixture of the following composition: 0.1 M Bis-Tris (pH 6.4) buffer, 0.67 μM S-adenosyl-L-[methyl-3H]methionine (Perkin Elmer, 78 Ci/mmol), and 2.9 μg human recombinant L-isoaspartyl methyltransferase (this enzyme preparation had a specific activity of 19.5 nmol min⁻¹ mg⁻¹ when the peptide KASA-isoD-LAKY was used as a substrate). The reaction mixtures were incubated for 2 h at 30°C and were quenched via addition of 50 μl 2X Laemmli sample buffer containing 10% β-mercaptoethanol followed by heating at 70°C for 8 min. Approximately 40 μg of the polypeptides within the above reaction mixtures were then separated by gel electrophoresis using NuPAGE® Novex 4–12% SDS-PAGE gels (pH = 7) (Catalog # NP0335) and NuPAGE® MES SDS Running Buffer (Catalog #NP002) from Invitrogen. The level of base-labile [3H]-methyl esters was assayed in 3 mm gel slices by a vapor diffusion method as described by Kagan et al. [24], except that 150 μl of 1 N sodium hydroxide were added to the gel slices and that scintillation vials were incubated at 38°C for 24 hours before counting. Methyl-accepting proteins were quantified in two or three biological replicate extracts for each worm strain tested.

Results

PCM-1 overexpression extends lifespan in a DAF-16-dependent manner

It has previously been shown that PCM-1 overexpression extends the lifespan of adult nematodes under mild thermal stress [17]. Nematodes expressing a PCM-1 protein with a mutated AdoMet-binding site in a pcm-1 null background (PL54 strain) had a lifespan that was similar to wild-type (N2) animals under the same conditions indicating that the lifespan extension of the overexpressor (PL51) strain was dependent on the methyltransferase activity of PCM-1 [17]. We confirm here that the lifespan extension observed in the PL51 strain is dependent on PCM-1 overexpression, as the lifespan of PL51 adult nematodes fed with two different clones of pcm-1 RNAi was comparable to that of wild-type nematodes treated with control RNAi (Figure S1). The lifespan of wild-type nematodes was not significantly affected by pcm-1 RNAi.

To test whether the PCM-1 overexpression effect was due to a modulation of the IIS system, we tested the dependence of this effect on the presence of DAF-16, the critical downstream transcription factor of the IIS. Specifically, we grew the N2, PL51, and PL54 strains on bacteria expressing scrambled control RNAi or daf-16 RNAi and used FUDR to prevent egg laying and cross generation sampling [29,36]. We confirmed that PCM-1 overexpressing animals display on average an increase of 38% and 51% in median and maximum lifespan, respectively, compared to wild-type animals, and that no change in survival is observed between wild-type nematodes treated with control RNAi (Figure S1). The lifespan of wild-type nematodes was not significantly affected by pcm-1 RNAi.

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Figure 1. PCM-1 overexpression extends lifespan in a daf-16 dependent manner under mild thermal stress (25°C). Lifespan analyses (three trials) were completed at 25°C for three nematode strains (N2 wild-type (top panel), wild-type PCM-1 overexpressor strain (PL51) (middle panel), mutant PCM-1 overexpressor strain (PL54) (bottom panel)) grown in two conditions: control RNAi (solid lines) and daf-16 RNAi (dotted lines). L4 larvae were transferred (Day 0) to NGM plates streaked with RNAi bacteria and survival was scored every other day until all nematodes were dead. Statistical analysis of these data is given in Table 2.
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Table 2. PCM-1 overexpression extends C. elegans lifespan in a DAF-16-dependent manner under mild thermal stress (25°C).

| Strain          | n  | 50% Survival (Hours) | 25% Survival (Hours) | 10% Survival (Hours) | Maximum Lifespan (Hours) |
|-----------------|----|---------------------|----------------------|---------------------|--------------------------|
| N2              | 152| 2.3±0.9             | 3.2±0.8              | 4.2±1.1             | 5.0±0.8                  |
| pcm-1 (qa201)   | 136| 1.7±0.6 (p = 0.04)**| 2.6±0.6 (p = 0.003)**| 3.3±1.0 (p = 0.01)**| 4.0±1.0                  |
| PL51            | 142| 3.7±0.6             | 4.5±0.5 (p = 0.04)*  | 5.2±0.6             | 6.0±0.8                  |
| PL54            | 123| 2.7±1.0             | 3.3±1.1              | 3.9±1.2             | 4.7±1.2                  |

Survival from the data in Figure 1 was determined using the one-way ANOVA test. One asterisk (*) significance compared to N2 control RNAi and two asterisks (**) significance compared to PL51 control RNAi denote statistical significance at the indicated p-value. The total number of adult nematodes (n) scored for survival in three replicates is indicated. Values represent the day (± standard deviation) at which specified survival for each strain was observed.

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values) measured for the overexpressor strain were statistically significant when compared to wild-type (N2) animals (Table 2).

We found that methyltransferase-induced increase of survival was greatly limited by the RNAi knockdown of daf-16. Although small lifespan decreases were observed in wild-type and PL54 strains upon daf-16 RNAi feeding as compared to control RNAi, a much more pronounced reduction in survival was measured in the PL51 overexpressor strain in those conditions (Fig. 1 and Table 2). In the PL51 strain, median and maximum lifespans over wild-type values were reduced from 38% and 51% on control RNAi to only 10 and 22% on daf-16 RNAi. These results show that the lifespan extension observed in PCM-1 overexpressing animals is at least partially dependent on daf-16 expression levels.

PCM-1 overexpression enhances resistance to severe thermal stress

In addition to displaying extended adult lifespans, daf-2 pathway mutants also show resistance to a variety of stressors, including severe thermal stress [32]. In testing the PL51 PCM-1 overexpressor strain for resistance to heat stress, we found that day 1 adults displayed increased thermotolerance at 37°C compared to wild-type (N2) animals, most significantly at the 25% survival time point (Fig. 2, Table 3). PL51 adult animals displayed 25% survival at 4.5±0.5 h compared to 3.2±0.8 h in N2 animals. The enhanced thermotolerance appeared to be at least partially dependent on a functional methyltransferase cofactor-binding domain, as the PL54 animals did not show significantly increased survival at any noted time point. Furthermore, pcm-1 mutant animals displayed a significant decrease in thermotolerance when compared to PL51 animals at all 50%, 25%, and 10% survival time points (Table 3).

Preliminary experiments using daf-16 RNAi indicates that the increased resistance to acute thermal stress of the PL51 overexpressor strain is, unlike the lifespan extension at 25°C, DAF-16 independent (Figure S2). To investigate this DAF-16 dependency under thermal stress further, we examined the effect of PCM-1 deficiency on the subcellular localization of this transcription factor at 25°C and 37°C. TJ356 nematodes (which express a GFP-tagged DAF-16 protein) were fed bacteria expressing scrambled control RNAi or pcm-1 RNAi. Following incubation at 25°C for 48 hours or at 37°C for two hours, the cellular localization of DAF-16 was observed (Figure S3). In our experiments at 25°C, DAF-16::GFP gave a diffuse signal indicating its presence in the cytosol as well as in the nucleus, concurring with results shown recently by Kawli and colleagues [37]. No difference in DAF-16 subcellular localization was observed between control RNAi- or pcm-1 RNAi-fed animals. This is in agreement with the absence of a lifespan phenotype of the pcm-1 mutant at 25°C. On control RNAi at 37°C, DAF-16 expression was mostly nuclear, confirming previous studies [28] (Figure S3). A similar nuclear localization was observed for DAF-16 on pcm-1 RNAi after incubation at 37°C (Figure S3). Thus, PCM-1 deficiency does not affect DAF-16 localization under thermal stress. This indicates that the decreased resistance of the pcm-1 mutant to acute thermal stress that we observe here is not mediated by DAF-16. Further work will be needed to determine whetherPCM-1 overexpression, unlike PCM-1 deficiency, affects DAF-16 localization.

PCM-1 overexpression activates DAF-16-dependent gene expression

Since the results from our lifespan analyses under mild thermal stress showed that the extended lifespan in PCM-1 overexpressing animals is dependent on daf-16 expression, we next wanted to determine whether PCM-1 overexpression modulates transcription of DAF-16 target genes previously linked to nematode longevity. cDNA was synthesized from total RNA extracted from synchronized day 1 adults (grown at 25°C) and was used to measure expression levels of pcm-1, daf-16, five genes previously shown to be up-regulated by DAF-16 (sod-3, hsp-12.6, dod-3, F21F3.3, and mlt-1) [35,38], and two genes previously shown to be down-regulated by DAF-16 (dod-22 and dod-24) [35,38] by real-time
qPCR. Transcript levels for these experimental genes were normalized to the transcript levels of two reference genes (cdcl42 and ama-1) in all of the cDNA samples included in the study. 

| Strain          | n   | 50% Survival (Hours) | 25% Survival (Hours) | 10% Survival (Hours) | Maximum Lifespan (Hours) |
|-----------------|-----|----------------------|----------------------|----------------------|--------------------------|
| N2              | 152 | 2.3±0.9              | 3.2±0.8              | 4.2±1.1              | 5.0±0.8                  |
| pcm-1 (qa201)   | 136 | 1.7±0.6 (p = 0.04)** | 2.8±0.6 (p = 0.003)**| 3.3±1.0 (p = 0.01)** | 4.0±1.0                  |
| PL51            | 142 | 3.7±0.6              | 4.5±0.5 (p = 0.04)** | 5.2±0.6              | 6.0±0.8                  |
| PL54            | 123 | 2.7±1.0              | 3.3±1.1              | 3.9±1.2              | 4.7±1.2                  |

Significance of survival from the data in Figure 2 was determined using a one-way ANOVA test. One asterisk (*, significance compared to N2) and two asterisks (**, significance compared to PL51) denote statistical significance at the indicated p-value. The total number of adult nematodes (n) scored for survival in four replicates is indicated. Values represent the hour (± standard deviation) at which specified survival for each strain was observed. doi:10.1371/journal.pone.0020850.t003

Table 3. PCM-1 overexpression enhances resistance to severe thermal stress (37°C) in C. elegans.

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Figure 3. PCM-1 modulates expression levels of DAF-16 target genes at 25°C. Fold changes for the expression of various genes in nematode strains PL51 (PCM-1 overexpressor), PL54 (mutant PCM-1 overexpressor), and pcm-1 (go201) mutant (pcm-1 null) versus wild-type (N2) are shown. Total RNA was extracted from animals grown at 25°C. Relative transcript levels versus N2 are shown for pcm-1 and daf-16 genes (panel A), DAF-16 up-regulated genes (panel B), and DAF-16 down-regulated genes (panel C). Amo-1 and cdc-42 were used as endogenous reference genes for normalization of the transcript levels of all of the genes involved in this expression study. Normalized -fold changes and statistical significance were determined using the Qiagen REST 2009 program (*p≤0.05, **p≤0.001, ***p≤0.0001) as shown in Table IV.

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C. elegans do not present any lifespan phenotype at either 20°C or 25°C; these results indicate that isoaspartyl damage might not affect lifespan in nematodes. In all of our labeling experiments we generally observed a major peak of radioactive methyl group incorporation at 21 kDa, but the level of this incorporation seemed to be independent of PCM-1 activity.

We also measured isoaspartyl levels in protein extracts derived from PCM-1 overexpressing nematodes at both 20°C or 25°C. Our results showed no decrease in base-labile [3H]-methyl ester groups below basal wild-type levels in the PCM-1 overexpressing (PL51) extracts derived from nematodes grown at either 20°C or 25°C. On the contrary, equal or variably increased amounts of damaged proteins in PL51 animals versus wild-type animals were observed (data not shown). This indicates that a several-fold increase in isoaspartyl methyltransferase activity over wild-type activity levels does not decrease isoaspartyl levels below those accumulating in wild-type animals and suggests that the lifespan extension displayed by PCM-1 overexpressing animals does not result from a decrease in overall protein damage (in agreement with the above conclusion that increased protein damage does not affect lifespan).

Discussion

We show here that PCM-1 overexpression extends nematode lifespan under mild thermal stress (25°C) in a DAF-16-dependent manner. This finding is in agreement with previous results indicating that PCM-1 activity in adult nematodes might be dispensable under control conditions, but becomes critically important under various stress conditions or in specific larval stages [17,25,26]. Similarly, in preliminary experiments carried out in preparation of this study, no lifespan extension over wild-type was observed in PCM-1 overexpressing nematodes when grown at 20°C.

By employing daf-16 RNAi, we were able to show that a reduction in daf-16 expression partially reduced the lifespan extension displayed by the PL51 overexpressor strain at 25°C. Daf-16 RNAi also decreased lifespan in the mutant overexpressor animals, but this reduction was much less pronounced and mimicked the one observed in wild-type animals. A small negative effect of daf-16 RNAi on lifespan in N2 animals has been reported previously [40,41]. Our results thus indicate that the PCM-1 isoaspartyl methyltransferase extends lifespan at 25°C at least partially by directly or indirectly downregulating the IIS pathway and thereby promoting nuclear translocation of DAF-16 with concomitant up-regulation of target genes promoting survival under mild thermal stress (Figure 5).

The partial nature of the reduction of the PCM-1-dependent lifespan extension by daf-16 RNAi suggests that PCM-1 might also act independently of daf-16 to increase lifespan. Possible targets include the transcription factor SKN-1 [42,43]. SKN-1 functions in parallel to DAF-16 to promote longevity and is also a direct target of IIS; in response to oxidative or xenobiotic stress, SKN-1 induces a set of stress response genes and reduced IIS leads to nuclear accumulation and transcriptional activation of SKN-1 in the intestine [42]. Alternatively, complete suppression of the lifespan extension may require daf-16 down-regulation also in the neurons. It has been shown that RNAi does not penetrate neurons effectively in C. elegans animals [44,45]; we have confirmed this result here by finding that daf-16 RNAi treatment does not affect DAF-16 expression in neurons. Although DAF-16 activation occurs in both neuronal and intestinal cells, it has been shown that DAF-16 activity specifically in the intestine increases the lifespan of daf-2;daf-16 double mutants substantially [46,47]. However, it has also been shown that lifespan extension in age-1 mutants requires both neuronal and intestinal DAF-16 activity [47,48].
Table 4. PCM-1 modulates expression of DAF-16 target genes under mild thermal stress (25°C).

| Strain                        | Gene       | Fold Change Versus N2 | Fold Change Versus N2 | Fold Change Versus N2 | Fold Change Versus N2 | Average Fold Change | Standard Deviation | 95% Confidence Interval | P-value (Versus N2) | P-value (Versus pcm-1 qa201) |
|-------------------------------|------------|------------------------|------------------------|------------------------|------------------------|---------------------|--------------------|------------------------|---------------------|------------------------|
|                               |            | Trial #1               | Trial #2               | Trial #3               | Trial #4               |                     |                    |                        |                     |                        |
| PL51 overexpressor strain     | pcm-1      | 28.0                   | 22.0                   | 41.1                   | 30.4                   | 9.8                 | 9.6 – 98.5         | 0.03*                  |                     |                        |
|                               | daf-16     | 0.9                    | 0.5                    | 3.7                    | 1.7                    | 1.8                 | 0.02 – 135.6       | 0.9                    |                     |                        |
| Daf-16 up-regulated Genes     | sod-3      | 27.0                   | 14.4                   | 17.7                   | 19.7                   | 6.5                 | 6.5 – 65.3         | 0.0001***              |                     |                        |
|                               | hsp-12.6   | 5.0                    | 2.6                    | 5.7                    | 4.4                    | 1.6                 | 1.7 – 11.2         | 0.03*                  |                     |                        |
|                               | dod-3      | 26.0                   | 2.2                    | 3.9                    | 16.0                   | 12.0                | 11.2 – 35.9        | 0.001**                |                     |                        |
|                               | F21F3.3    | 55.7                   | 3.2                    | 2.1                    | 11.0                   | 18.0                | 25.4 – 69.9        | 0.06                   |                     |                        |
|                               | mtl-1      | 24.3                   | 1.9                    | 3.4                    | 11.3                   | 10.2                | 10.2 – 28.4        | 0.07                   |                     |                        |
| Daf-16 down-regulated Genes   | dod-22     | 0.2                    | 0.7                    | 0.4                    | 0.4                    |                     |                    |                        |                     |                        |
|                               | dod-24     | 0.4                    | 0.8                    | 0.6                    | 0.3                    |                     |                    |                        |                     |                        |
| PL54 mutant overexpressor strain | pcm-1     | 0.9                    | 1.0                    | 0.5                    | 0.8                    | 0.3                 | 0.42 – 1.2         | 0.5                    | 0.09                 |                        |
|                               | daf-16     | 0.8                    | 0.6                    | 1.8                    | 1.1                    | 0.6                 | 0.02 – 163.3       | 0.8                    | 0.09                 |                        |
| Daf-16 up-regulated Genes     | sod-3      | 3.3                    | 2.8                    | 1.0                    | 2.4                    | 1.2                 | 0.9 – 4.5          | 0.1                    | 0.05                 |                        |
|                               | hsp-12.6   | 4.6                    | 9.4                    | 1.5                    | 5.2                    | 4.0                 | 1.6 – 10.8         | 0.04*                  | 0.3                  |                        |
|                               | dod-3      | 2.5                    | 0.3                    | 1.7                    | 2.6                    | 2.3                 | 0.3 – 5.9          | 0.3                    | 0.1                  |                        |
|                               | F21F3.3    | 9.2                    | 1.0                    | 4.4                    | 12.1                   | 6.7                 | 0.6 – 19.4         | 0.06                   | 0.004*               |                        |
|                               | mtl-1      | 2.0                    | 0.3                    | 1.0                    | 9.2                    | 3.1                 | 4.1                | 0.20 – 8.4            | 0.06                 | 0.03*                 |
| Daf-16 down-regulated Genes   | dod-22     | 8.3                    | 5.3                    | 6.8                    | 2.1                    |                     |                    |                        |                     |                        |
|                               | dod-24     | 5.5                    | 6.9                    | 6.2                    | 1.0                    |                     |                    |                        |                     |                        |
| pcm-1 (qa201) mutant strain   | pcm-1      | 0.001                  | 0.006                  | 0.000                  | 0.002                  | 0.003               | 0.000 – 0.006      | 0.03*                  |                     |                        |
|                               | daf-16     | 0.04                   | 1.0                    | 14.6                   | 5.2                    | 8.1                 | 0.04 – 29.7        | 0.9                    |                     |                        |
| Daf-16 up-regulated Genes     | sod-3      | 0.6                    | 3.3                    | 0.7                    | 1.5                    | 1.5                 | 0.3 – 3.1          | 0.9                    |                     |                        |
|                               | hsp-12.6   | 1.3                    | 7.3                    | 1.2                    | 3.3                    | 3.5                 | 0.8 – 6.9          | 0.1                    |                     |                        |
|                               | dod-3      | 0.3                    | 0.2                    | 0.8                    | 0.4                    | 0.3                 | 0.2 – 1.1          | 0.2                    |                     |                        |
|                               | F21F3.3    | 0.04                   | 0.1                    | 0.03                   | 0.1                    | 0.0                 | 0.008 – 0.2        | 0.0001***              |                     |                        |
|                               | mtl-1      | 0.2                    | 0.1                    | 0.3                    | 0.2                    | 0.1                 | 0.06 – 0.6         | 0.0001***              |                     |                        |
| Daf-16 down-regulated Genes   | dod-22     | 0.01                   | 0.04                   | 0.04                   | 0.0                    | 0.0                 | 0.005 – 0.2        | 0.0001***              |                     |                        |
|                               | dod-24     | 1.3                    | 11.3                   | 3.7                    | 5.4                    | 5.2                 | 0.9 – 11.1         | 0.2                    |                     |                        |

Normalized relative transcript levels for the indicated genes and worm strains compared to wild-type (N2) animals were determined after growth at 25°C. *Ama-1 and cdc-42 were used as endogenous reference genes for normalization. Fold changes and statistical parameters were calculated using the Qiagen REST 2009 program (*p<0.05, **p<0.001, ***p<0.0001). doi:10.1371/journal.pone.0020850.t004
Thus, it is possible that the small lifespan extension we observe with PCM-1 overexpression in the *daf-16* RNAi treated adult nematodes is a result of DAF-16 activation in neurons. It is also known that PCM-1 is expressed primarily in the neurons, body wall, and reproductive tissues in *C. elegans* [25]. We thus speculate that a complete suppression of the *daf-16*-dependent lifespan extension triggered by PCM-1 overexpression might only be achievable in neuronal RNAi-sensitive worm strains.

We also observed an increase in median survival under severe thermal stress (37°C) in the overexpressor strain that is dependent on a functional L-isoaspartyl methyltransferase. Preliminary examination of the enhanced thermal stress resistance in the *PL51* strain indicates that the increased thermotolerance observed is *daf-16*-independent (Figure S2). In agreement with this, our preliminary microscopy results show that *pcm-1* RNAi does not suppress nuclear localization of DAF-16 during heat shock at 37°C (Figure S3). Thus, increased survival of the *PL51* nematodes at 37°C might involve a DAF-16-independent mechanism.

Real-time qPCR analyses showed a PCM-1-dependent induction of the DAF-16 target genes *sod-3, F21F3.3, mtl-1*, and *dod-3* at 25°C. The 20-fold induction of *sod-3* transcription and the approximately 10-fold to 20-fold induction of the *dod-3, F21F3.3, and mtl-1* genes over wild-type levels in the PCM-1 overexpressor strain at 25°C further solidifies our hypothesis of PCM-1 down-regulating IIS signaling to extend nematode longevity under mild thermal stress. We note that *mtl-1* expression can also be regulated by the ELT-2 transcription factor [49]. At this point, we cannot rule out effects of PCM-1 on DAF-16-independent pathways. Although the bulk of our evidence points to the linkage of PCM-1 activity to the activity of DAF-16, it is possible that PCM-1 can affect DAF-16 target genes through other transcription factors.

Surprisingly, the mutant overexpressor strain *PL54* did not overexpress *pcm-1* to the same extent as the wild-type overexpressor strain *PL51*. We confirmed the presence of the expected point mutations in the *pcm-1* transcript in the *PL54* strain, and consequently believe that the mutant overexpressor strain expresses a non-functional PCM-1 protein at lower levels, as our activity assays confirm that there is no PCM-1 activity in this strain. It was interesting to observe that a few of the “DAF-16 up-regulated” genes were also significantly up-regulated compared to wild-type animals in the *PL54* mutant overexpressor strain. Except for *hsp-12.6*, for which an approximate 5-fold induction was observed in both *PL54* and *PL51* strains, the levels of induction of the other DAF-16 up-regulated genes were, however, much lower in the *PL54* mutant overexpressor strain than those reached in the *PL51* wild-type overexpressor strain. These effects may be attributed to the regulation of *hsp-12.6* by the HSF-1 transcription factor (50) or by effects of the *C10F3.4/mcp-1* gene which overlaps the *pcm-1* gene in antiparallel orientation and which is overexpressed to the same extent in both the *PL51* and *PL54* transgenic strains.

Concerning the “DAF-16 down-regulated” genes analyzed in our expression study (*dod-22* and *dod-24*), the results obtained in the PCM-1 transgenic strains (about 2-fold down-regulation in the *PL51* strain as opposed to an about 6- to 7-fold up-regulation in...
the PL54 strain) also support our hypothesis of a PCM-1 mediated downregulation of the IIS. However, whereas the dod-24 gene was up-regulated to a similar extent in the PL54 mutant overexpressor strain as in the pcm-1 deletion strain, a more puzzling result was the very low expression measured for dod-22 in the latter strain. It might be noted here that the status of the dod-22 and dod-24 genes as “downstream of DAF-16” targets is presently controversial. Initial work by Murphy and colleagues [38] identified dod-22 and dod-24 as genes acting downstream of DAF-16 to regulate longevity in C. elegans. These genes were also shown to be down-regulated in long-lived TGF-β mutant adult animals and this regulation was dependent on DAF-16 [51]. Additionally, the dod-22 and dod-24 genes were used as control down-regulated DAF-16 target genes in a recent study on HCF-1, a negative regulator of DAF-16 [35]. Other recent studies [52,53] suggest, however, that regulation of dod-22 and dod-24 expression is largely independent of DAF-16. Dod-24 expression was shown to be negatively regulated by the nuclear hormone receptor DAF-12 via signaling from the somatic reproductive tissues and independently of DAF-16 [48] and dod-22 expression has been shown to be regulated by the p38/MAPK pathway independently of DAF-16 [53]. Both studies show only a partial/restrictive dependence on DAF-16, explaining their presence in the original screen [38]. Given those recent observations on the regulation of dod-22 and dod-24 expression, the effects of PCM-1 on the expression levels of those genes might not, or only be partially mediated by DAF-16. Further work is needed to clarify this point.

To determine whether an increase in PCM-1 activity leads to increased protein repair in C. elegans, we measured protein isoaspartyl levels in wild-type, pcm-1 mutant, and PL51 extracts by using human recombinant isoaspartyl methyltransferase. Our results show an increased isoaspartyl content in pcm-1 mutant extracts compared to wild-type extracts prepared from nematodes grown in liquid culture, suggesting a defect in the repair of isoaspartyl residues in the absence of methyltransferase. In the PCM-1 overexpressing (PL51) extracts, there was no apparent decrease in isoaspartyl damage compared to wild-type levels. However, PL51 is a non-integrated transgenic strain, with an observed transmission of about 30%. Thus, we estimate that the liquid cultures from which we prepared our extracts contained

Figure 5. The protein L-isoaspartyl O-methyltransferase (PCM-1) opposes insulin-like signaling to extend C. elegans lifespan. Upon binding of the insulin-like ligand to the DAF-2 receptor, the AGE-1 kinase is activated and phosphorylates membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP2), leading to the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and recruitment of kinases PDK-1 and AKT-1/AKT-2/S GK-1 complex to the membrane. PDK-1 subsequently phosphorylates and activates the AKT/S GK complex, leading to further phosphorylation and inactivation of the FoxO transcription factor DAF-16. In the absence of phosphorylation, DAF-16 enters the nucleus to induce transcription of genes implicated in stress resistance and longevity. Our results indicate that overexpression of the protein repair enzyme PCM-1 leads to down-regulation of the insulin-like signaling pathway and subsequent activation of DAF-16, leading to increased transcription of longevity genes. Our results also show that, although absence of PCM-1 activity leads to increased accumulation of protein damage, its overexpression might not decrease the level of isoaspartyl-containing proteins beyond wild-type levels. This suggests that PCM-1 might control lifespan in C. elegans through a regulatory signaling function rather than through its role in bulk protein repair.

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about 30% PCM-1 overexpressing transgenic animals and 70% non-transgenic pcm-1 mutant animals. Hence, if the transgenic PCM-1 overexpressing animals had decreased levels of isoaspartyl damage, this change may have been masked by the increased levels of damage in the 70% non-transgenic pcm-1 mutant nematodes.

Taken together, these results suggest that increased accumulation of protein damage (as observed in mutant pcm-1 animals) does not affect lifespan (no difference in lifespan between wild-type and pcm-1 mutant animals) and, conversely, that PCM-1 overexpression might not extend lifespan by decreasing the overall content of damaged proteins in the cell. Rather, PCM-1 may act as a signaling protein to directly or indirectly downregulate the IIS pathway to turn on expression of stress resistance genes via activation of DAF-16 (Figure 5). One possible mechanism could be a posttranslational regulatory carboxyl methylation of an intermediate of the IIS or of a protein phosphatase by PCM-1. The future identification of such potential regulatory targets of PCM-1 will help elucidate the mechanism of the observed interaction between the isoaspartyl methyltransferase, the IIS, and lifespan control in nematodes as well as in higher organisms.

Supporting Information

Figure S1  Pcm-1 RNAi reduces the lifespan extension of PCM-1 overexpressing adult nematodes at 25°C. Lifespan analyses (one trial) were completed at 25°C (with FUDR) for two nematode strains (N2 wild-type (top panel) and wild-type PCM-1 overexpressor strain [PL51] (bottom panel) grown in three conditions: control RNAi (solid lines) and pcm-1 RNAi (clones #2 and #3, dotted lines). L4 larvae were transferred (Day 0) to NGM plates streaked with RNAi bacteria and survival was scored every other day until all nematodes were dead. Wild-type (N2) animals scored for survival were as follows: control (n = 74) and pcm-1 RNAi conditions (n = 77 for pcm-1 RNAi clone #2; n = 76 for pcm-1 RNAi clone #3). PL51 animals scored for survival were as follows: control (n = 55) and pcm-1 RNAi conditions (n = 70 for pcm-1 RNAi clone #2; n = 70 for pcm-1 RNAi clone #3).

(TIF)

Figure S2  Daf-16 RNAi does not significantly affect resistance to severe heat stress (37°C) in PCM-1-overexpressing animals. Survival assays (one trial) were completed for two nematode strains (N2 wild-type and PCM-1 overexpressor strain [PL51]) in control RNAi and daf-16 RNAi conditions. L4 larvae were transferred to NGM + RNAi plates and were allowed to grow at 20°C overnight. The next day, animals were transferred to 37°C and survival was scored every two hours for the first four hours and every hour afterwards until all nematodes were dead. Wild-type (N2) animals scored for survival were as follows: control (n = 27) and daf-16 RNAi conditions (n = 26). PL51 animals scored for survival were as follows: control (n = 15) and daf-16 RNAi conditions (n = 20).

(TIF)

Figure S3  Monitoring DAF-16 localization with pcm-1 RNAi under mild (25°C) and severe (37°C) thermal stress. TJ356 nematode animals were fed bacteria expressing either control (scramble) or pcm-1 RNAi as described in the Methods sections. DAF-16 localization was monitored using Nomarski differential interference contrast microscopy at 200-fold magnification. The top three panels show mostly diffuse, unlocal-ized DAF-16 expression in animals fed control RNAi (n = 22), pcm-1 RNAi clone #2 (n = 25), and pcm-1 RNAi clone #3 (n = 19) at 25°C. The bottom three panels show nuclear DAF-16 expression in animals fed control RNAi (n = 8), pcm-1 RNAi clone #2 (n = 12), and pcm-1 RNAi clone #3 (n = 13) following incubation at 37°C.

(PDF)

Figure S4  Quantification of protein isoaspartyl levels in additional C. elegans extracts at 20°C. Protein extracts were prepared from liquid nematode cultures grown at 20°C and analyzed for isoaspartyl content as described in Figure 4. Data from two additional biological replicate lysates are shown in panel A (replicate 1) and panel B (replicate 2). For panel A, base-labile [3H]-methyl ester groups present in peak 22 (not visible in figure) for N2+ hrPCM and pcm-1 (qa201) + hrPCM are 14,514 cpm and 12,862 cpm, respectively. For panel C, base-labile [3H]-methyl ester groups present in peak 26 (not visible in figure) for N2+ hrPCM and pcm-1 (qa201) + hrPCM are 247 cpm and 380 cpm, respectively. A less active human recombinant PCM was used in replicate 2 compared to the hrPCM used in the experiments shown in Figure 4 and in panel A of this Figure, but the same increase in base-labile [3H]-methyl ester groups in labeled pcm-1 (qa201) mutant extracts was observed.

(TIF)

Figure S5  Quantification of protein isoaspartyl levels in additional C. elegans extracts prepared from nematodes grown at 20°C (top panel) and 25°C (bottom panel). Protein extracts were prepared from liquid nematode cultures and analyzed for isoaspartyl content as described in Figure 4. The averages of data from two additional biological replicate lysates (prepared from nematodes grown at 20°C) are shown in the top panel. Base-labile [3H]-methyl ester groups present in peak 23 (not visible in figure) for N2+ hrPCM and pcm-1 (qa201) + hrPCM are 7,851 cpm and 19,384 cpm, respectively. In the bottom panel, data from two additional biological replicate lysates (prepared from nematodes grown at 25°C) for both strains are shown. Base-labile [3H]-methyl ester groups present in peak 24 (not visible in figure) for N2+ hrPCM and pcm-1 (qa201) + hrPCM are 4,858 cpm and 8,017 cpm, respectively.

(TIF)

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Author Contributions

Conceived and designed the experiments: SK CLL SGC. Performed the experiments: SK CLL. Analyzed the data: SK CLL SGC. Wrote the paper: SK CLL SGC.

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