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The PTEN tumor suppressor homolog in *Caenorhabditis elegans* regulates longevity and dauer formation in an insulin receptor-like signaling pathway

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ABSTRACT **Inactivation of the tumor suppressor *PTEN* gene is found in a variety of human cancers and in cancer predisposition syndromes. Recently, PTEN protein has been shown to possess phosphatase activity on phosphatidylinositol 3,4,5-trisphosphate, a product of phosphatidylinositol 3-kinase. We have identified a homolog of PTEN in *Caenorhabditis elegans* and have found that it corresponds to the *daf-18* gene, which had been defined by a single, phenotypically weak allele, *daf-18(e1375)*. By analyzing an allele, *daf-18(nr2037)*, which bears a deletion of the catalytic portion of CePTEN/DAF-18, we have shown that mutation in *daf-18* can completely suppress the dauer-constitutive phenotype caused by inactivation of *daf-2* or *age-1*, which encode an insulin receptor-like molecule and the catalytic subunit of phosphatidylinositol 3-kinase, respectively. In addition, *daf-18(nr2037)* dramatically shortens lifespan, both in a wild-type background and in a *daf-2* mutant background that normally prolongs lifespan. The lifespan in a *daf-18(nr2037)* mutant can be restored to essentially that of wild type when combined with a *daf-2* mutation. Our studies provide genetic evidence that, in *C. elegans*, the PTEN homolog DAF-18 functions as a negative regulator of the DAF-2 and AGE-1 signaling pathway, consistent with the notion that DAF-18 acts a phosphatidylinositol 3,4,5-trisphosphate phosphatase *in vivo*. Furthermore, our studies have uncovered a longevity-promoting activity of the PTEN homolog in *C. elegans*.**

Human PTEN, also called MMAC1 or TEP1, is encoded by a tumor suppressor gene located on chromosome 10q23 (1–3). Mutation or deletion of the *PTEN* gene has been found in a variety of human cancers, such as glioblastoma, endometrial tumors, and prostate cancer and in familial cancer predisposition syndromes (4). Recently, it has been shown that mice carrying a homozygous *PTEN* gene deletion are embryonic lethal, whereas mice heterozygous for the deletion show hyperproliferation in the prostate, skin, and colon, reminiscent of the features of human Cowden's disease and Bannayan–Ruvalcaba–Riley syndrome (5, 6). These studies indicate that PTEN is a *bona fide* tumor suppressor.

The PTEN protein contains a sequence motif, HCXXGXGRXG, that is highly conserved among members of the protein tyrosine phosphatase family. PTEN protein can dephosphorylate phosphotyrosyl and phosphoseryl/threonyl residues on protein substrates *in vitro* (3, 7). Recently, it has been shown that PTEN can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3) (8). PIP3 is generated by phosphatidylinositol 3-kinase (PI 3-kinase), which is activated by binding to ligand-engaged receptor tyrosine kinases (9). PIP3 then acts as a second messenger to activate a variety of signaling molecules, one of which is Akt. Akt, also called PKB, is a serine/threonine kinase whose activation requires binding to PIP3 through its pleckstrin homology domain and subsequent recruitment to the plasma membrane (10). In human tumor cells, overexpression of PTEN leads to decreased levels of PIP3 and inactivation of Akt (11–17). Conversely, in mouse $Pten^{-/-}$ cells in which the *Pten* gene has been genetically deleted, increased levels of PIP3 and enhanced activation of Akt are observed (18, 19). Mouse $Pten^{-/-}$ cells show decreased apoptosis (18, 19) and accelerated cell cycle progression (19). These studies suggest that mammalian PTEN functions as a phosphatase for the inositol phospholipid PIP3. The role of PTEN as a tumor suppressor has been attributed to its ability to modulate two important cellular processes: cell cycle progression and apoptosis. However, it remains unclear whether PIP3 is the major *in vivo* substrate for PTEN, because dephosphorylation of protein substrates (e.g., FAK, a protein tyrosine kinase associated with focal adhesions) or inhibition of the mitogen-activated protein kinase pathway have also been reported (20, 21). In addition, the lethality of the homozygous *PTEN* gene deletion in mice makes it difficult to carry out a detailed analysis of the biological processes affected by the complete PTEN deficiency in mammals.

Caenorhabditis elegans provides a model system in which powerful genetic analysis can be used to investigate the major signaling pathways and the physiological processes regulated by a PTEN homolog in a living organism. Here we report that the *C. elegans* homolog of the mammalian PTEN tumor suppressor is involved in dauer formation and lifespan regulation and that CePTEN is encoded by the *daf-18* gene. The dauer state is essentially a state of hibernation that is normally only entered under conditions of starvation or overcrowding. The insulin receptor-like protein DAF-2 and the PI 3-kinase homolog AGE-1 are also known to function in both dauer formation and lifespan (22, 23). Inactivation of either *daf-2* or *age-1* causes animals to form dauer larvae rather than progressing continuously through the four larval stages and into adulthood (24). In addition, *daf-2* or *age-1* animals that reach adulthood have a dramatically extended lifespan, indicating that signaling triggered by the insulin receptor-like tyrosine kinase has a profound influence on the aging process in *C. elegans* (22, 25–28). Both the constitutive dauer formation and lifespan extension phenotypes observed in *daf-2* or *age-1* mutants requires the activity of *daf-16*, which encodes two fork–head group transcription factors (25, 27, 29–32). Another gene that has been suggested to function downstream of *daf-2* and *age-1* is *daf-18* (27, 28, 30). The sole original mutation, *daf-18(e1375),* was isolated as a mutant that failed to form dauer larvae even under starvation conditions (33). Studies of *daf-18* have been hampered by the fact that this sole allele only partially suppresses certain *daf-2* or *age-1* mutant phenotypes, and because this allele by itself has a weak phenotype (27–30, 33).

We have genetically characterized a *daf-18* mutation that contains a deletion in the phosphatase catalytic domain of CePTEN/DAF-18. Our results establish the role of the *C. elegans*

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Abbreviations: PIP3, phosphatidylinositol 3,4,5-trisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase.

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PTEN homolog in the *daf-2* and *age-1* signaling pathway that regulates both longevity and dauer development.

MATERIALS AND METHODS

Strain Constructions. The *daf-18(nr2037)* mutation was backcrossed five times against the wild-type Bristol N2 strain. The presence of the *nr2037* allele was identified by PCR analysis of genomic DNA using the primers F6 (5′-CTATTGAAGGAGGACTAACACAGGC-3′) and R3 (5′-GCCAACGAAGT-GCTAAATCGAC-3′). A diagnostic 0.6-kb or 1.6-kb fragment is indicative of the presence of the *nr2037* or wild-type allele, respectively. The presence of the wild-type allele was further confirmed by PCR using the primers F3 (5′-GATTGGTGTCTACGTGGAACGG-3′) and R3, which produce a 0.8-kb fragment only in the wild-type strain. The *daf-2(e1370); daf-18(nr2037)* double mutant strain was constructed by crossing *daf-18(nr2037)* males with *daf-2(e1370)* hermaphrodites. Homozygous *daf-2(e1370)* F_2 progeny were identified by their dauer-constitutive phenotype at 25°C. These animals were allowed to exit from the dauer state by shifting to 20°C, and progeny were identified as *daf-18(nr2037)* homozygotes by using PCR. Sequencing of genomic DNA confirmed that the *daf-2(e1370)* allele was, in fact, homozygous. To construct the *age-1(m333); daf-18(nr2037)* double mutant, *daf-18(nr2037)* males were mated with *age-1(m333)/mnC1[dpy-10(e128) unc-52(e444)]* hermaphrodites. F_2 progeny that were *age-1/age-1; daf-18/+* were identified based on their ability to produce progeny consisting of dauers and very slowly growing non-dauers at a 3:1 ratio. The slow-growing non-dauers were *age-1(m333); daf-18(nr2037)* candidates containing maternally provided *daf-18(+)* activity. In the next generation, the maternal *daf-18(+)* contribution was absent, and the progeny regained a normal growth rate. These animals were confirmed to be *daf-18(nr2037)* homozygotes by using PCR analysis. The presence of the *age-1(m333)* allele in the double mutant strain was confirmed by backcrossing with wild-type males and observing the reappearance of the *age-1(m333)* dauer-constitutive phenotype in some of the descendents of all cross-progeny. Two independent strain isolates were used to assay lifespan and dauer phenotype for all strains constructed in this study, and similar results were obtained for both isolates in all cases. The following strains were obtained from the *C. elegans* Genetic Center: CB1370, *daf-2(e1370)*; DR722, *age-1(m333)/mnC1[dpy-10(e128) unc-52(e444)]*; and CB1375, *daf-18(e1375).*

Allelic Sequencing. Genomic DNA was amplified by using PCR, and the PCR fragments were directly sequenced. For all of the alleles, sequencing was performed on both DNA strands. The *daf-18(nr2037)* allele contains a deletion that removes nucleotides 650–1,639 (in reference to the initiation codon as +1). *daf-18(e1375)* contains a 30-bp insertion at nucleotide 2,852. This mutation was found in strain CB1375.

cDNA Characterization. cDNA clones for *daf-18* (yk400b8, yk43e5, and yk181h9) were obtained from Yuji Kohara (National Institute of Genetics, Mishima, Japan). The cDNA clone with the largest insert, yk400b8, was fully sequenced.

Genomic Rescue. A 13.6-kb *Bam*HI–*Sal*I genomic fragment, containing 1.3 kb of upstream sequence, 5 kb of *daf-18* coding region, and 7.3 kb of downstream sequence, was derived from cosmid T07A9. The Δ*Bsp*HI derivative was constructed by deleting the internal 620-bp *Bsp*HI–*Bsp*HI fragment, resulting in the removal of exons I and II of the *daf-18* gene. Each construct was injected into the *daf-2(e1370); daf-18(nr2037)* double mutant strain at 25 ng/μl together with the transformation marker plasmid pRF4 (100 ng/μl) (34). Stable transgenic lines were obtained by following germ-line transmission of the Roller phenotype. Multiple independent transgenic lines have been established for both *daf-18(+)* or *daf-18(−)* transgenes. Progeny from these stable lines were used to examine *daf-18* rescue activity by scoring the reappearance of the dauer phenotype at 25°C. Data were pooled from three independent stable lines for each transgene.

Lifespan Assay. Lifespan experiments were performed essentially as described (25). For assays performed at 25°C, eggs were hatched and raised at 15°C. At the L4 or young adult stage, animals were transferred to new plates and shifted to 25°C. The day of the temperature shift was counted as day 0 in the lifespan assay. For assays performed at 20°C, eggs were hatched and raised at 20°C. At the L4 or young adult stage, animals were transferred to new plates (day 0) and maintained at 20°C. Animals were transferred to new plates once every day during their reproductive period and then once every 3 days after the end of their egg-laying period. The animals were scored as dead the day they failed to respond to a light touch with a platinum wire. The *daf-18(nr2037)* mutant has a low penetrance protruding vulva phenotype (≈8%) which can lead to vulval ruptures and the death of the animal. For other strains, animals with progeny that hatched internally were observed at low frequencies. All of these animals were excluded in the final data analysis for survival curve and mean lifespan, because they obviously did not die of old age.

Scoring Dauer Phenotypes. The dauer-constitutive phenotype of *daf-2(e1370)* and *age-1(m333)* mutants were scored as described (27, 30). For the *daf-2(e1370)* or *daf-18(nr2037)* single mutants, *daf-2(e1370); daf-18(nr2037)* double mutant, and the derived transgenic lines, eggs were laid at 20°C during a 10-hour period and shifted to 25°C. Progeny were scored at 48 hours after the temperature shift and then rescored 24 hours later to confirm the phenotype. For the *age-1(m333)* single mutant and the *age-1(m333); daf-18(nr2037)* double mutant, eggs were layed at 20°C during a 10-hour period and maintained at 20°C. Progeny were scored at 48 hours and again at 72 hours after egg-laying. The categories for dauers and partial dauers were defined as described (35).

RESULTS

***C. elegans* Homolog of PTEN.** We have identified a homolog of PTEN in the *C. elegans* sequence database generated by the *C. elegans* Genome Sequencing Consortium (T07A9.6). The DNA sequence of a full-length cDNA clone (yk400b8) revealed a predicted ORF of 962 aa, abbreviated as CePTEN. The genomic structure of the *CePTEN* gene is shown in Fig. 1*A*. The amino-terminal region of CePTEN contains the phosphatase catalytic domain, and this region shares 38% sequence identity with the corresponding domain of human PTEN (Fig. 1*B*). The carboxyl-terminal region of CePTEN has lower levels of sequence homology to human PTEN. The noncatalytic domains of CePTEN and PTEN may play a species-dependent regulatory role.

To understand the function of CePTEN, we employed a reverse genetics approach. A deletion mutation in the *CePTEN* gene, *nr2037*, was identified by using PCR screening from an ordered array of mutagenized worms (kindly provided by Carl Johnson and Leo Liu; NemaPharm, Cambridge, MA). Sequencing of the *nr2037* allele revealed a deletion that removes 990 nt of the *CePTEN* gene spanning parts of intron 2 and exon 3 (Fig. 1*A*). The *nr2037* mutation is predicted to abolish the phosphatase activity of CePTEN, because the deletion removes part of the catalytic domain, including the conserved sequence motif, HCKAGKGRTG, that forms the phosphatase catalytic center. The rest of exon 3 also is unlikely to be translated because of removal of the splice acceptor site at the 5′ end of exon 3.

Strains homozygous for *nr2037* are viable and show no obvious abnormalities in development or morphology when grown under well fed conditions. However, at high-saturation growth densities or under starvation conditions, we noticed that *nr2037* homozygotes failed to form dauers (a dauer-defective phenotype). The dauer-defective phenotype can be rescued by germ-line transformation with a small genomic fragment containing the wild-type *CePTEN* gene (see below), indicating that this phenotype results from the *nr2037* lesion. These observations indicate that the *CePTEN* gene is required for dauer larvae formation. Because the PI 3-kinase pathway is known to be involved in dauer formation and because our parallel biochemical studies in mam-

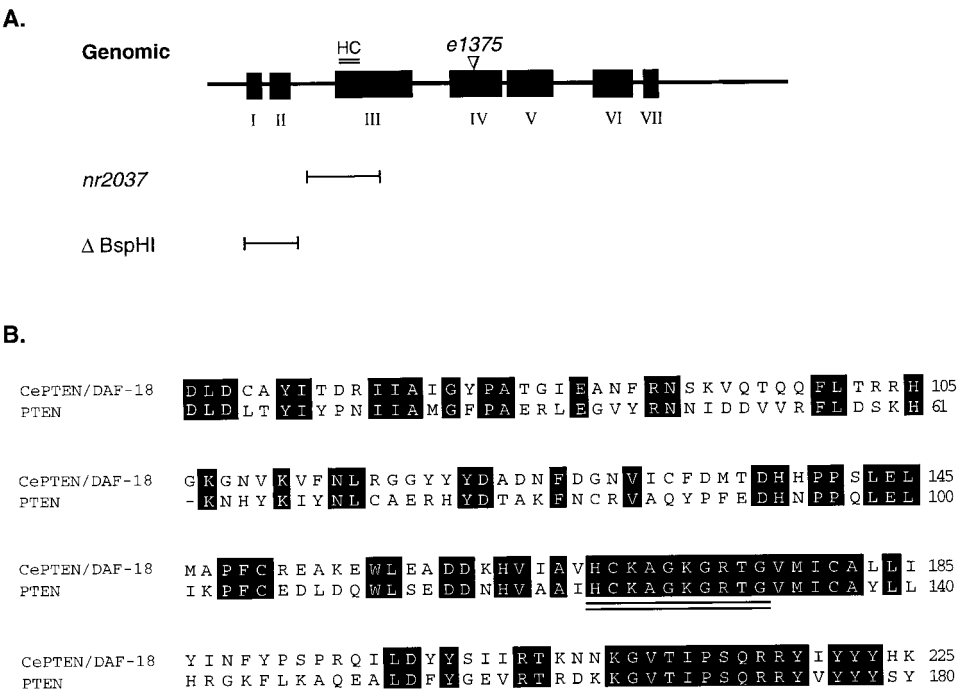

FIG. 1. *CePTEN/daf-18* gene structure. (*A*) Genomic structure. The exons are depicted as black boxes and numbered. The *nr2037* allele contains a deletion that covers part of intron II and exon III. The phosphatase catalytic center, HCKAGKGRTG, abbreviated as the HC motif (double underlined), is deleted in the *nr2037* mutant. The Δ*Bsp*HI deletion construct, in which exon I and II of the *CePTEN/daf-18* gene is removed, is used as a control for the genomic rescue experiment (see Table 1). The 30-bp insertion found in the *daf-18(e1375)* allele is located in exon IV in the noncatalytic domain of CePTEN/DAF-18. This insertion occurs after codon 574, leading to a frameshift and premature stop codon. (*B*) Amino acid alignment of the amino-terminal phosphatase domain of CePTEN/DAF-18 and human PTEN. The phosphatase catalytic center, HCKAGKGRTG, is double underlined. Identical residues are shown as white letters in black boxes.

malian cells have suggested that PTEN acts as a phosphatase for PIP3 (16, 19), we more closely examined *nr2037* mutant animals for phenotypes known to be affected by PI 3-kinase signaling, as described below.

CePTEN Is Encoded by the *daf-18* Gene. The dauer-defective phenotype of *nr2037* animals resembles the phenotype described for *daf-18(e1375)* (33). *daf-18* maps to chromosome IV, in close proximity to the *CePTEN* gene locus. To determine whether the *daf-18(e1375)* mutation also affects the *CePTEN* gene, we sequenced the genomic region of the *CePTEN* locus in *daf-18(e1375)* animals and found a 30-bp insertion in exon IV (see Fig. 1*A*). This insertion occurs downstream of the phosphatase catalytic domain and leads to premature termination of the CePTEN protein. We have adopted the nomenclature DAF-18 for CePTEN and *daf-18(nr2037)* for the *nr2037* allele. The nature of the *daf-18(e1375)* mutation was recently independently reported by Ogg and Ruvkun (36).

The molecular nature of the *nr2037* and *e1375* mutations suggests that the *nr2037* mutation should completely eliminate the phosphatase activity of CePTEN/DAF-18, whereas the *e1375* mutation may only partially reduce CePTEN/DAF-18 function. As described below, our detailed genetic characterization of *daf-18(nr2037)* strongly supports such a hypothesis.

***daf-18(nr2037)* Suppresses the Dauer-Constitutive Phenotype of *daf-2(e1370)*.** The *daf-2* and *age-1* signaling pathway is known to be required for normal development through the third larval stage (L3 stage). In molecular terms, appropriate levels of PIP3, produced through the PI 3-kinase AGE-1 and its upstream regulator DAF-2 (a receptor tyrosine kinase of the insulin receptor subfamily), are required for prevention of dauer formation. *daf-2(e1370)* is a temperature-sensitive mutant that forms dauer larvae when grown at the nonpermissive temperature of 25°C (a temperature-sensitive, dauer-constitutive phenotype) (27, 29). We reasoned that if CePTEN/DAF-18 acts as a phosphatase for PIP3 *in vivo*, loss of CePTEN/DAF-18 might promote accumulation of PIP3 and balance the effect of insufficient production of PIP3 caused by inactivation of DAF-2. Consequently, in *daf-2; daf-18* double mutants, wild-type levels of PIP3 may be restored, thus allowing normal larval development instead of dauer arrest. We therefore constructed the *daf-2(e1370); daf-18(nr2037)* double mutant strain and tested whether *daf-18(nr2037)* could suppress the dauer-constitutive phenotype of *daf-2(e1370)* at the nonpermissive temperature of 25°C. As shown in Table 1, *daf-2(e1370)* homozygotes form dauer larvae at 100% penetrance in this assay. These dauers are characterized by nonfeeding, reduced locomotion, a dark intestine, and a thin body appearance. In addition, *daf-2(e1370)* dauer larvae have a characteristic remodeled pharynx (Fig. 2). By contrast, *daf-2(e1370); daf-18(nr2037)* double mutants develop normally through the L3 stage and into adulthood when assayed in parallel (Table 1). These double mutants do not show the remodeled pharynx that is characteristic of dauer larvae (Fig. 2). The *daf-18(nr2037)* single mutant also develops normally and has a normal pharynx (Table 1 and Fig. 2). The complete suppression of the *daf-2(e1370)* dauer-constitutive phenotype at 25°C by the *daf-18(nr2037)* mutation suggests that *CePTEN/daf-18* acts in an antagonistic manner to *daf-2*.

We have used the suppression of *daf-2(e1370)* to confirm that the phenotypic effects that we observed for *daf-18(nr2037)* are due to the deletion in the *CePTEN* gene. We have established stable transgenic lines by using a small genomic fragment that carries essentially only the wild-type *CePTEN/daf-18* gene. This genomic fragment rescued the *daf-18(nr2037)* defect in the *daf-2(e1370); daf-18(nr2037)* double mutant background, as the presence of the wild-type *CePTEN/daf-18* transgene allowed the reappearance of the *daf-2(e1370)* dauer-constitutive phenotype at 25°C [Table 1, transgene *daf-18(+)*]. As a control, stable lines established by using a genomic fragment in which exons 1 and 2 of the *CePTEN/daf-18* gene were deleted showed no rescue activity when assayed in parallel [Table 1, transgene *daf-18(−)*]. Nomarski microscope examination of pharyngeal morphology corroborated these results (Fig. 2). These studies showed that the wild-type, but not mutant, *CePTEN/daf-18* transgene can completely rescue the *daf-18(nr2037)* defect, indicating that the phenotype we observed for *daf-18(nr2037)* is indeed due to the loss of *CePTEN/daf-18* gene activity.

***daf-18(nr2037)* Suppresses the Dauer-Constitutive Phenotype of *age-1(m333)*.** We next asked whether reduced production of PIP3, caused by inactivation of the AGE-1 PI 3-kinase, could be compensated, at least partially, by loss of the putative PIP3 phosphatase CePTEN/DAF-18. *age-1* activity is known to be required for normal development through the L3 stage (22, 27). Maternally provided *age-1(+)* activity is sufficient to allow *age-1/age-1* homozygotes to progress through larval stages to adulthood. However, in the absence of the maternal *age-1(+)* contribution, *age-1/age-1* homozygotes arrest as dauer larvae uncon-

Table 1. The *daf-18(nr2037)* mutation suppresses the dauer-constitutive phenotype of *daf-2(e1370)*

Genotype of parent	Transgene	Phenotype of progeny at 25°C, %				
		L4 and adult	Dauer	Partial dauer	Other	*n*
Wild type	none	100	0	0	0	250
daf-2(e1370)	none	0	100	0	0	376
daf-18(nr2037)	none	98.5	0	0	1.5	200
daf-2(e1370); daf-18(nr2037)	none	96.5	0	0	3.5	310
daf-2(e1370); daf-18(nr2037)	*daf-18(+)*	0	93.5	4.3	2.2	203
daf-2(e1370); daf-18(nr2037)	*daf-18(−)*	99	0	0	1.0	205

Eggs were collected at 20°C during a 10-hour period and then shifted to 25°C. Phenotypes were scored at 48 hours after the temperature shift and confirmed by rescoring 24 hours later. Partial dauers are defined as those with less constriction or with less darkness of the intestine than typical dauer progeny observed in the *daf-2(e1370)* strain. "Other" includes animals that died as young larvae or those with grossly abnormal morphology. Wild type, Bristol N2 strain. *n*, total number of animals scored.

Stable transgenic lines were established using the wild-type genomic *daf-18* fragment, *daf-18(+)*, or the same fragment with a deletion of exons 1 and 2, *daf-18(−)*. Transgene data are the combined total from three independent lines.

ditionally (22, 27). We wondered whether the dauer-constitutive phenotype caused by complete loss of *age-1* could be suppressed by the *daf-18(nr2037)* mutation, and we therefore constructed the *age-1(m333); daf-18(nr2037)* double mutant. As shown in Table 2, in the absence of any maternal *age-1* contribution, *age-1/age-1* homozygotes form dauers at 100% penetrance. By contrast, *age-1(m333); daf-18(nr2037)* double mutants develop normally past the L3 stage to adulthood. This is also true for *age-1(m333); daf-18(nr2037)/daf-18(e1375)* trans-heterozygotes, further confirming the allelism of these two mutations (data not shown). The fact that the *age-1(m333)* dauer arrest phenotype can be completely suppressed by the *daf-18(nr2037)* mutation provides genetic evidence that CePTEN/DAF-18 opposes the action of AGE-1.

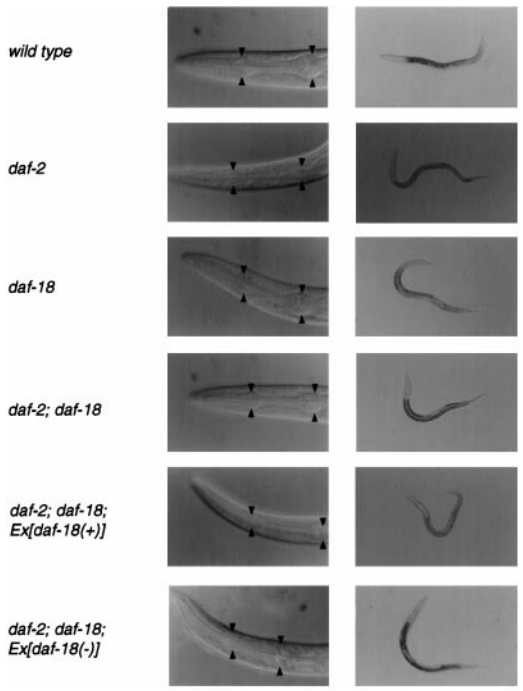

FIG. 2. Morphological comparison of *daf-18*, *daf-2*, and *daf-2; daf-18* strains. (*Left*) Nomarski micrographs. The head of the animal is shown. The two bulbs of the pharynx are indicated by the arrowheads. (*Right*) Brightfield micrographs. Transgenic animals, shown in the *Bottom* two rows, carry an extrachromosomal array of either the functional *daf-18* gene (*Ex*[*daf-18(+)*]), or a mutant derivative that removes exons I and II (*Ex*[*daf-18(−)*]). The animals with genotypes *daf-2(e1370)* and *daf-2(e1370); daf-18(nr2037); Ex*[*daf-18(+)*] are dauer larvae and appear thinner in the brightfield micrographs and have a constricted pharynx as shown in the Nomarski micrographs. Wild-type animals and animals with genotypes *daf-18(nr2037)*, *daf-2(e1370); daf-18(nr2037)* and *daf-2(e1370); daf-18(nr2037); Ex*[*daf-18(-)*] are non-dauer L3 larvae.

***daf-18(nr2037)* Shortens the Lifespan of *C. elegans*.** The ability of the *daf-18(nr2037)* mutation to suppress the *daf-2(e1370)* dauer-constitutive phenotype is in contrast to the previous report that the *daf-18(e1375)* mutation could not suppress *daf-2(e1370)* in such an assay (27, 29). These differences are likely due to *daf-18(nr2037)* being a null allele, whereas *daf-18(e1375)* only partially reduces gene function, as one would predict from the molecular nature of these mutations (see Fig. 1). We therefore tested the effect of the stronger *daf-18(nr2037)* mutation on the lifespan of animals, because the aging process in *C. elegans* is known to be affected by PI 3-kinase signaling. Our experiments showed that at 25°C, *daf-18(nr2037)* mutants have a shorter lifespan than wild-type animals (Fig. 3*A*). The mean lifespan is 6.2 days for *daf-18(nr2037*) as compared to 8.5 days for wild type, and the reduction of lifespan by the *daf-18(nr2037)* mutation is ≈30% (Table 3). The maximum lifespan of *daf-18(nr2037)* animals is also shorter than that of the wild type, 9 days versus 14 days (Table 3).

When we performed the similar experiment at 20°C, the optimal temperature to culture *C. elegans*, the lifespan curve of the *daf-18(nr2037)* mutant was further separated from that of the wild-type strain (Fig. 3*B*). The mean lifespan for *daf-18(nr2037)* is 5.8 days, whereas the wild type's lifespan is 12.7 days (Table 3). The mean lifespan of the *daf-18* mutant is 50% shorter than that of the wild type. These studies demonstrate that CePTEN/DAF-18 has a normal function in preventing the onset of aging.

Comparing Fig. 3 *A* and *B*, the lifespan curves for *daf-18(nr2037)* at 25°C and 20°C are almost superimposable. By contrast, the lifespan curve of the wild-type strain is shifted to the left at 25°C. In other words, the lifespan of the wild-type strain is considerably shortened at 25°C, whereas the lifespan of the *daf-18(nr2037)* mutant is largely unaffected. As discussed below, we suggest that the temperature effect on lifespan may be due to increased production of the inositol phospholipid PIP3 at higher temperature.

Suppression of the *daf-18(nr2037)* Lifespan Shortening Phenotype by Mutation in *daf-2*. We hypothesized that the lifespan-shortening phenotype caused by the *daf-18(nr2037)* mutation is

Table 2. The *daf-18(nr2037)* mutation suppresses the dauer-constitutive phenotype of *age-1(m333)*

Genotype of parent	Phenotype of Progeny at 20°C, %				
	L4 and adult	Dauer	Partial dauer	Others	*n*
age-1(m333)	0	100	0	0	410
age-1(m333); daf-18(nr2037)	100	0	0	0	320

Eggs were collected at 20°C during a 10-hour period and maintained at 20°C. Phenotypes were scored twice, first at 48 hours, and then again at 72 hours after egg laying. Homozygous *age-1(m333)/age-1(m333)* hermaphrodites were obtained from *age-1(m333)/mnC1*[*dpy-10(e128) unc-52(e444)*] parents. Definitions for the partial dauers or others are as in Table 1. *n*, total number of animals scored.

due to accumulation of higher levels of PIP3 as a result of the loss of the candidate PIP3 phosphatase CePTEN/DAF-18. Accordingly, a decrease in the production of PIP3 caused by inactivation of the DAF-2 receptor tyrosine kinase might be able to restore normal levels of PIP3 in a *daf-18(nr2037)* background and consequently extend lifespan. We thus compared the lifespan of the *daf-18(nr2037); daf-2(e1370)* double mutant with that of the *daf-18(nr2037)* single mutant, the *daf-2(e1370)* single mutant, and the wild-type strain at 25°C and 20°C. As shown in Fig. 3 and Table 3, the *daf-2(e1370)* mutation almost doubled the lifespan of the animals, consistent with previous reports (25). Interestingly, we found that the *daf-2(e1370)* mutation extended the lifespan of *daf-18(nr2037)* animals at either 25°C or 20°C and restored lifespan to that found for wild type (Fig. 3 *A* and *B*, Table 3). These observations suggest that the lifespan-shortening phenotype caused by *daf-18(nr2037)* depends on the positive signal input from *daf-2* gene activity. In addition, *daf-18(nr2037)* also completely suppressed the long-lived phenotype of *daf-2(e1370)*. The lifespan of the *daf-2(e1370); daf-18(nr2037)* double mutant is almost identical to that of the wild-type strain at both 25°C and 20°C (Fig. 3, Table 3). The mutual suppression of the *daf-18(nr2037)* and *daf-2(e1370)* alleles suggests that their corresponding gene products function in opposite directions in regulating lifespan in *C. elegans*.

DISCUSSION

In *C. elegans*, DAF-2, an insulin receptor-like molecule, and AGE-1, a PI 3-kinase homolog, are involved in a signaling pathway that negatively regulates lifespan and the dauer-formation processes. Our genetic studies have shown that DAF-18, the *C. elegans* homolog of PTEN, functions in an antagonistic manner to the actions of DAF-2 and AGE-1 (Fig. 4). Our studies strongly support the hypothesis that CePTEN/DAF-18 acts as a physiological phosphatase for PIP3.

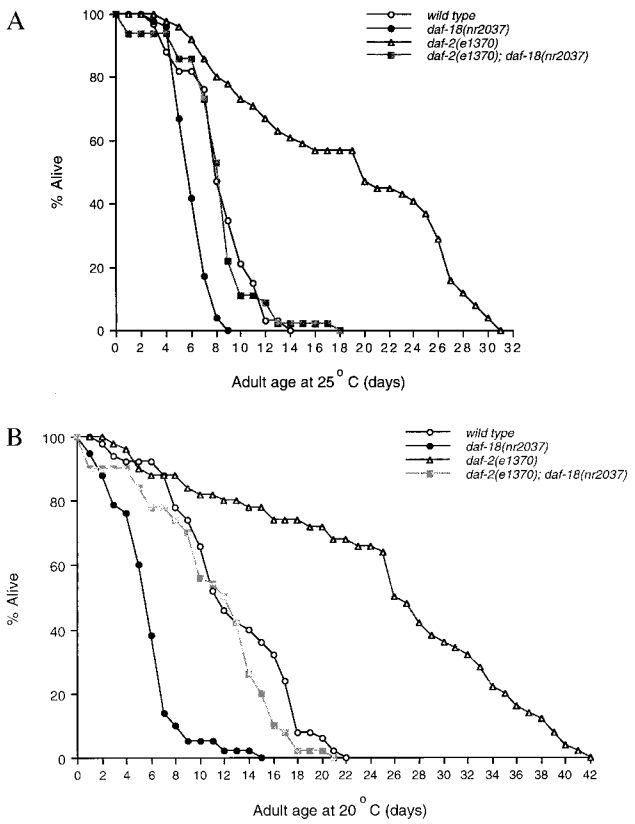

FIG. 3. Effects of the *daf-18(nr2037)* mutation on adult lifespans. (*A*) Lifespans at 25°C. (*B*) Lifespans at 20°C. The percentage of live animals was plotted as a function of time (days). Wild-type (Bristol N2), *daf-18(nr2037)*, *daf-2(e1370)*, and *daf-2(e1370); daf-18(nr2037)* strains were compared in parallel. Representatives of two independent sets of experiments are shown. The results were similar in both sets of experiments.

Table 3. Effect of the *daf-18(nr2037)* mutation on adult lifespans

Strain	Lifespan, days Mean (±SE)	Ratio vs. wildtype	Maximum	*n*
25°C*				
Wild type	8.5 (±0.3)	1.0	14	34
daf-18(nr2037)	6.2 (±0.2)	0.7	9	48
daf-2(e1370)	18.8 (±0.5)	2.2	31	49
daf-2(e1370); daf-18(nr2037)	8.3 (±0.4)	1.0	18	49
20°C†				
Wild type	12.7 (±0.3)	1.0	22	50
daf-18(nr2037)	5.8 (±0.5)	0.5	15	42
daf-2(e1370)	25.3 (±0.5)	2.0	42	50
daf-2(e1370); daf-18(nr2037)	11.1 (±0.5)	0.9	21	50

Values are calculated using the data from the experiments shown in Fig. 3. The relative ratio of the mean over that of the wild-type (Bristol N2) strain is also shown. All experiments were performed at least two times with similar results obtained. *n*, total number of animals scored.

*The animals were raised at 15°C until the L4 or adult stage and then shifted to 25°C. The day of the shift is counted as day 0 in the adult lifespan assay.

†The animals were raised and maintained at 20°C. Young adults or L4s were transferred to new plates to assay for lifespan. The day of the transfer is counted as day 0 in the adult lifespan assay.

We have analyzed a mutant allele of *daf-18*, *nr2037*, that is likely to eliminate all *daf-18* function. The *nr2037* allele consists of a deletion that removes the phosphatase catalytic domain and likely causes frameshifts of the rest of the protein. This mutation completely suppresses the dauer-constitutive phentotype conferred by a mutation that compromises the function of *daf-2*. Our studies thus suggest that the production of PIP3 is an essential portion of the DAF-2 signaling pathway for prevention of dauer formation. This is in contrast to the previous reports that the *daf-18(e1375)* mutation could not suppress the dauer-constitutive phenotype conferred by *daf-2(e1370)* (27, 29) and that *daf-18* RNA interference could only partially suppress the *daf-2(e1370)* dauer phenotype (36). These differences in the ability to suppress the effect of the *daf-2(e1370)* mutation could result from a more complete elimination of DAF-18 activity by *daf-18(nr2037)* than that caused by either the *daf-18(e1375)* mutation or *daf-18* RNA interference. In contrast with its ability to suppress *daf-2(e1370), daf-18(e1375)* could completely suppress the effects of null alleles of *age-1* (e.g., *m333* or *mg44* allele) on dauer development (27, 30), suggesting that the reduced *daf-18* gene activity caused by the *daf-18(e1375)* mutation is sufficient to restore appropriate PIP3 levels for normal larval development, even in the complete absence of AGE-1 activity. The inability of *daf-18(e1375)* to suppress *daf-2(e1370)* may be due to a potential negative regulation of *daf-18* by *daf-2* (illustrated in Fig. 4). Alternatively, it is possible that the signaling pathway bifurcates downstream of DAF-2, with AGE-1/PI 3-kinase being only one of the pathways involved. If DAF-2 normally inhibits DAF-18 activity, as shown in Fig. 4, in the *daf-2(e1370); daf-18(e1375)* strain background, the inactivation of *daf-2* will cause an increase of *daf-18(e1375)* gene activity. This increase will lead to further reduction of PIP3 levels, triggering dauer arrest. However, in the *age-1(m333); daf-18(e1375)* mutant strain, the *daf-2* gene activity is still intact, and can inhibit *daf-18(e1375)*. As a consequence, a more potent reduction of *daf-18(e1375)* activity is achieved, leading to a greater restoration of PIP3 levels and allowing the animal to proceed through normal larval development.

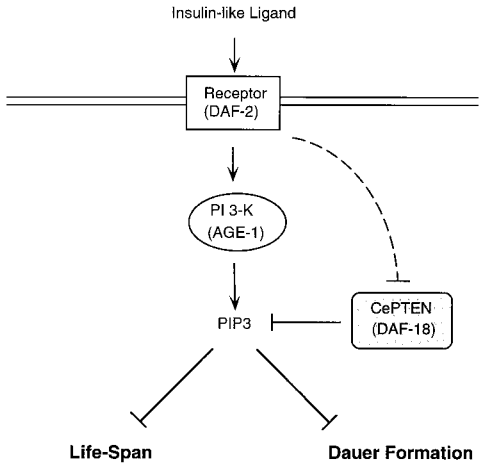

FIG. 4. Model for DAF-18 action. AGE-1 is the major signal transducer for the activated DAF-2 insulin receptor-like molecule in regulation of dauer development and lifespan. AGE-1 is predicted to produce PIP3, which negatively regulates lifespan and dauer-formation processes. DAF-18, the PTEN homolog, functions to dephosphorylate PIP3 and thus antagonizes the action of DAF-2 and AGE-1. DAF-18 may also be negatively regulated by DAF-2 (dashed line).

Previous genetic screens have failed to identify mutations in *daf-18* as suppressors of the *daf-2* dauer-constitutive phenotype. We can account for this based on our observations that maternal *daf-18* contributions provide sufficient activity to allow *daf-2(e1370); daf-18(nr2037)* double mutants to remain dauer-constitutive (data not shown). Such a property of *daf-18* may explain the failure to recover *daf-18* mutants in the extensive genetic screens performed to date, because many of these screens were not designed to uncover mutants that show maternal rescue activity.

Interestingly, we found that *daf-18(nr2037)* has profound effects on lifespan. Complementary to previous reports that reduced *daf-2* or *age-1* activity leads to doubling of the lifespan, we observed that *daf-18(nr2037)* causes up to 50% shortening of lifespan. In comparison, the *daf-18(e1375)* mutation is reported to have a very modest effect on lifespan, although it can suppress the lifespan extension phenotype caused by *daf-2* mutation (27, 28). Such differences could also be attributed to differences in the severity of the *daf-18* alleles being assayed. Accordingly, a higher steady-state level of PIP3 in the *daf-18(nr2037)* mutant may be accumulated than that in the *daf-18(e1375)* mutant, which in turn leads to the premature aging that we observe. It is interesting to note that in wild-type animals, the onset of aging is accelerated by exposure to higher temperature, as shown by the shift of the survival curve toward shorter lifespans and by the reduction of the mean lifespan of animals at 25°C versus 20°C. Such a shift may be due to increased PIP3 production at higher temperature. The lifespan of the *daf-2(e1370)* single mutant and the *daf-2(e1370); daf-18(nr2037)* double mutant also showed temperature dependence. However, in the *daf-18(nr2037)* single mutant, higher temperature has very little effect on lifespan. It is likely that in the *daf-18(nr2037)* strain, even at 20°C, the PIP3 level is already above the threshold that is required to activate the aging process. Temperature-dependent production of PIP3 cannot further accelerate this process.

In summary, we have shown that DAF-18, the *C. elegans* homolog of the PTEN tumor suppressor, functions as a negative regulator of the *daf-2* and *age-1* signaling pathway. This is consistent with the model that CePTEN/DAF-18 functions as a PIP3 phosphatase *in vivo*. Our studies have also uncovered CePTEN/DAF-18 as one of the rate-limiting factors that control the onset of aging in *C. elegans*. Our results thus raise an interesting possibility that in addition to its tumor suppressor function, mammalian PTEN may function to promote longevity in a signal-transduction pathway activated by insulin-like growth factors.

Note Added in Proof. While this manuscript was being reviewed, Gil *et al.* also reported the characterization of the *daf-18 (nr2037)* allele in the dauer pathway (37).

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