Regulation of the insulin-like developmental pathway of *Caenorhabditis elegans* by a homolog of the *PTEN* tumor suppressor gene

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ABSTRACT   The human *PTEN* tumor suppressor gene is mutated in a wide variety of sporadic tumors. To determine the function of PTEN *in vivo* we have studied a *PTEN* homolog in *Caenorhabditis elegans*. We have generated a strong loss-of-function allele of the *PTEN* homolog and shown that the deficient strain is unable to enter dauer diapause. An insulin-like phosphatidylinositol 3-OH kinase (PI3′K) signaling pathway regulates dauer-stage entry. Mutations in either the *daf-2* insulin receptor-like (IRL) gene or the *age-1* encoded PI3′K catalytic subunit homolog cause constitutive dauer formation and also affect the life span, brood size, and metabolism of nondauer animals. Strikingly, loss-of-function mutations in the *age-1* PI3′K and *daf-2* IRL genes are suppressed by loss-of-function mutations in the *PTEN* homolog. We establish that the *PTEN* homolog is encoded by *daf-18*, a previously uncloned gene that has been shown to interact genetically with the DAF-2 IRL AGE-1 PI3′K signaling pathway. This interaction provides clear genetic evidence that PTEN acts to antagonize PI3′K function *in vivo*. Given the conservation of the PI3′K signaling pathway between *C. elegans* and mammals, the analysis of *daf-18 PTEN* mutant nematodes should shed light on the role of human PTEN in the etiology of metabolic disease, aging, and cancer.

The human *PTEN* (also known as Mutated in Multiple Advanced Cancers 1) tumor suppressor gene was cloned because of its frequent mutation in late stage glioblastomas and prostate tumors (1, 2). Subsequent studies have identified *PTEN* mutations in a wide variety of sporadic human cancers, including endometrial tumors (3), malignant melanomas (4), and bladder carcinomas (5). Germ-line human PTEN mutations also have been identified as the underlying genetic lesions of both Cowden disease (6) and Bannayan–Zonana syndrome (7). These autosomal dominant syndromes result in a high predisposition to hamartomas, benign tumors that contain differentiated but disorganized cells. Cowden disease also is characterized by an increased incidence of breast and thyroid tumors. This predisposition to malignancies is recapitulated in mice carrying targeted mutations within the murine *PTEN* gene (8). *PTEN* heterozygous mutant mice develop dysplasias in a wide spectrum of tissues and have a high incidence of prostate and colon tumors (8, 9). *PTEN* homozygous mutant mouse embryos die between embryonic day 7.5 and 9.5 with patterning defects (8, 9), an overgrowth of cells in the caudal and cephalic regions of the embryo, and defects in placentation (9). Thus, *PTEN* is required for a number of developmental processes, but the mechanism by which it exerts its effects is unknown.

The predicted human PTEN protein sequence contains a 200-aa region called a TAG domain (10) that shares homology with tensin, auxilin, and G cyclin-associated kinase (1, 2). Nested within the TAG domain is a dual-specificity protein phosphatase signature motif (refs. 1 and 2; Fig. 1). *In vitro* studies have shown that human PTEN displays phosphatase activity against a number of substrates, including focal adhesion kinase (11). PTEN preferentially dephosphorylates phosphoamino acids surrounded by acidic residues (12). Consistent with this preference, PTEN can dephosphorylate the D3 position of the acidic substrate phosphatidylinositol (3,4,5) triphosphate (PtdIns-3,4,5-P_3; ref. 13), a product of phosphatidylinositol 3-OH kinase (PI3′K; ref. 14).

In *C. elegans*, a PI3′K signal transduction pathway modulates the ability of nematodes to enter the developmental dauer-larval stage (15). The insulin/IGF-I receptor-like (IRL; IGF-I is insulin-like growth factor I) molecule DAF-2 (16) transduces a signal via a PI3′K, AGE-1 (15), and two Akt homologues, AKT-1 and AKT-2 (17), to prevent dauer formation induced by the DAF-16 transcription factor (17–19). Loss-of-function mutations in *daf-2* IRL or *age-1* PI3′K lead animals to form dauers constitutively and arrest development (20, 21). In adult animals, disruption of *daf-2* IRL or *age-1* PI3′K increases fat storage as well as longevity (16, 22). The modulation of nematode metabolism by an insulin-like pathway is similar to the control of mammalian glucose homeostasis by insulin (16). This similarity suggests that evolutionarily conserved factors that impinge on the DAF-2 IRL AGE-1 PI3′K signaling cascade in *C. elegans* may play a causal role in human metabolic diseases, such as type II diabetes (16), in which insulin signaling is impaired.

To study the *in vivo* function of *PTEN*, we have identified a *C. elegans PTEN* homolog. We find that animals that carry a deletion in the *PTEN* gene are unable to form dauers and that this mutation suppresses mutations in *daf-2* IRL and *age-1* PI3′K. The *PTEN* homolog maps close to a gene known to be required for dauer formation, *daf-18* (20), and both sequence and genetic analyses indicate that *daf-18* encodes the *PTEN* homolog. Thus, the *C. elegans PTEN* homolog acts to antagonize signaling through the PI3′K pathway. Intriguingly, *daf-18 PTEN* mutant animals exhibit a number of secondary phenotypes that may be independent of the DAF-2 IRL AGE-1 PI3′K signaling cascade. This pleiotropy suggests that *PTEN* may play a role in a second signal transduction pathway *in vivo*.

Fig. 1. Sequence analysis of the *Caenorhabditis elegans PTEN* homolog and structure of the *nr2037* deletion allele. The *C. elegans PTEN* homolog from cosmid T07A9 is aligned with human PTEN. Identical amino acids are indicated in bold; identities and conservative changes are shaded. The phosphatase signature motif and probable catalytic aspartic acid are indicated by a box. Conserved tyrosine phosphorylation sites are indicated by asterisks. Amino acids encoded by sequences deleted in the *nr2037* allele are overlined. The deletion removes bp 1,991–2,980 according to GenBank's numbering of cosmid T07A9.

We discuss the implications of *C. elegans daf-18 PTEN* biology for cancer and type II diabetes.

MATERIALS AND METHODS

Isolation of a *PTEN* Deletion Mutant and Sequence Analysis of the *daf-18(e1375)* Allele. A library of *C. elegans* mutants was generated by trimethylpsoralen treatment and UV irradiation (L.X.L., J. Spoerke, E. Mulligan, J. Chen, B. Reardon, M. Basson, R. Clover, and C.D.J., unpublished work). Worm populations were arrayed in microtiter plates with 20 F_1 animals per well, and genomic DNA was sampled from pools of nematodes. This DNA was subjected to PCR analysis by using nested primers designed to assay for deletions in the phosphatase and TAG homology domain of the *PTEN* homolog. External primers were ATGCAGTGAGAAGAGAG-GCGTTCG and ACCGACTCCTCGAATATCTCCAC, and internal primers were ACCCGTGCCGTTTGAATTAGC and CCTGATTCCGTGTATGATTTGTCTC. A deletion amplicon of 2.2 kb (as opposed to a wild-type PCR product of 3.2 kb) was identified from a library of 192,000 genomes that had been exposed to mutagens. Sibling selection and single-worm PCR were used to isolate individual worms bearing the deletion mutation, which was given the allele name *nr2037*. The sequence of the *PTEN* homolog in *daf-18(e1375)* was determined by single-worm PCR amplification of exons and intron–exon junctions, followed by direct sequencing.

Strains and Maternal Rescue. The mutations used in this study were, for linkage group II, *age-1(m333, mg44)*, *sqt-1(sc13)*, and *mnC1[dpy10(e128)* and *unc-52(e444)]*; for linkage group III, *daf-2(e1370ts)*; and for linkage group IV, *daf-18(e1375* and *nr2037)* and *dpy9(e12)*. Mutations are described in ref. 23, except for *daf-18(nr2037)*, whose generation is described above. Strains were provided by the Horvitz lab (Massachusetts Institute of Technology, Cambridge, MA) or by the Caenorhabditis Genetics Center (St. Paul, MN). Single-worm PCR was performed for the *nr2037* deletion allele with the primers GATTGGTGTCTACGTGGAACGG,GCCAA-CGAAGTGCTAAATCGAC, and AATTCAATCGTCGA-GCGG. The combination of these three primers yielded wild-type amplicons of 718 bp and 2,077 bp and/or a deletion

amplicon of 1,087 bp. Single-worm PCR was performed for the *daf-18(e1375)* allele with the primers AAGTGTCGACATT-TGACGGCTCCTCTACTG and CTTCGAGATGTACTGC-GTTGC. The mutant amplicon was 28 bp larger than the wild-type amplicon and could be separated on a 3% agarose gel. Details concerning the construction of *daf-2;daf-18* or *age-1;daf-18* double-mutant animals are available on request. The presence of *daf-2* or *age-1* in the double-mutant animals was confirmed by complementation. Maternal rescue effects were delineated by inspecting the self-progeny of a*ge-1(m333)/ age-1(m333);daf-18/+* hermaphrodites.

Germ-Line Transformation. Germ-line transformation was performed as described in ref. 24, with DNA concentrations of 100 µg/ml or 20 µg/ml T078KBSP or 100 µg/ml pRF4 (see Table 3). T078KBSP contains an 8-kb *Sma*I–*Sac*II subclone of *C. elegans* cosmid T07A9. The pRF4 plasmid contains the *rol-6(su1006)* allele as a dominant marker (24). Rol progeny from injected adults were picked singly to plates, and lines that segregated the extrachromosomal arrays were used in the phenotypic tests.

Analysis of the Dauer-Defective Phenotype and Complementation Tests. For assay of dauer formation under starved, crowded conditions, animals were picked singly to a nematode growth media-agar plate. Animals were allowed to deplete the bacteria on the plate, and 5 days later, they were treated with 3 ml of 1% SDS. Plates were inspected visually 30–60 min later for live dauers. For complementation tests, *nr2037/+* males were mated to *e1375* homozygote hermaphrodites. Individual hermaphrodite progeny were assayed for dauer formation as above. Plates were scored for the presence of the *nr2037* deletion by PCR analysis of a "scoop" of progeny by using deletion-specific primers. Initial complementation tests used the DR235 strain, which has been designated *daf-18(e1375) dpy-9(e12)*. However, the DR235 and *daf-18(e1375)* (strain CB1375 from the Caenorhabditis Genetics Center) complemented each other in a dauer-formation test. Furthermore, DR235 animals did not burst. These results indicate that DR235 does not contain the *e1375* mutation.

Determination of Brood Size. L4 larvae were picked singly to a nematode growth media-agar plate and grown at 25°C. Adults were moved once a day for 3 days. Progeny were counted 40–48 h after eggs were laid. Numbers of progeny were counted on at least 4 days for each hermaphrodite parent in each experiment.

Analysis of the Dauer-Constitutive and Bursting Phenotypes. For each genotype, 1–10 adults were allowed to lay eggs for 5–6 h. Progeny were grown at 25°C and inspected visually for dauers, L4 larva, or adults 54–56 h later. Where applicable, progeny were inspected again at 78–80 h and 102–104 h later for dauer morphology or the bursting phenotype.

RESULTS

Mutation of the *C. elegans PTEN* Homolog Prevents Entry into the Dauer Stage. A search of A *Caenorhabditis elegans* Database (ACeDB) identified a homolog of the human PTEN gene on cosmid T07A9 (GenBank accession no. AF036706). The sequence of the genomic locus and that of a full-length cDNA (yk400b8, kindly provided by Y. Kohara, National Institute of Genetics, Mishima, Japan) predict a 962-aa protein (Fig. 1). The *C. elegans* protein is 43% identical to human PTEN over the 200-aa TAG domain and differs in only 1 of 11 residues in the phosphatase signature motif. There is significantly less homology in the C-terminal portion of these proteins, but a potential tyrosine phosphorylation site is conserved. This site is mutated specifically in a tumor-derived form of human PTEN, suggesting that it plays an important role in the regulation of this protein (2).

To isolate a defective *PTEN* allele, we used a PCR-based approach (ref. 25; L.X.L., J. Spoerke, E. Mulligan, J. Chen, B.

Reardon, M. Basson, R. Clover, and C.D.J., unpublished work) to screen a library of *C. elegans* mutants for animals carrying a deletion in the TAG/phosphatase domain. We identified a 990-bp deletion, designated *nr2037*, that spans sequences from intron 2 to exon 3 (Fig. 1). This deletion removes much of the TAG domain, including the entire phosphatase signature motif. We believe *nr2037* to be a null mutation of the *C. elegans PTEN* homolog. Animals carrying the *nr2037* deletion were outcrossed seven times and subjected to phenotypic analysis. These mutants were found to have a number of partially penetrant phenotypes. A small proportion of the *nr2037* animals was sterile, was defective in laying eggs, or had a tendency to form "bags-of-worms" (data not shown). In addition, 17% of these mutants burst from the vulva as adults (Table 1). None of the *nr2037* animals were able to form dauers, as judged by SDS resistance (Table 1). We therefore conclude that the *C. elegans PTEN* homolog is necessary for normal entry into dauer diapause.

The *C. elegans PTEN* Homolog Is a Component of the *age-1*/P13 Kinase Pathway. Entry into the *C. elegans* dauer stage is regulated by an IRL protein DAF-2 that transduces its signal through a PI3′K homolog, AGE-1 (15, 16). Mutation of either of these genes leads to constitutive dauer formation (20, 21). PI3′K is known to phosphorylate phosphatidylinositol (3, 4)bisphosphate (16, 26) to generate PtdIns-3,4,5-P_3 (14), and human PTEN can catalyze the reverse reaction *in vitro* (13). We hypothesized that the dauer-formation defect of *nr2037* mutant animals results from changes in the regulation of the *age-1* PI3′K pathway. To test this hypothesis, we examined the phenotype of animals with both the *nr2037* deletion mutation and a loss-of-function mutation in either *daf-2* IRL or *age-1* PI3′K. The *nr2037* deletion allele completely suppressed the dauer constitutive phenotype of these *daf-2* IRL and *age-1* PI3′K mutations (Table 2). *nr2037* also rescued the brood-size reduction that has been described for the *daf-2(e1370)* mutants (refs. 27 and 28; Table 2). We therefore conclude that the *PTEN* homolog functions either downstream of or parallel to *daf-2* IRL and *age-1* PI3′K *in vivo*. Because PtdIns-3,4,5-P_3 is both the product of PI3′K (14) and a target for dephosphorylation by PTEN (13), it is likely that the PTEN homolog contributes to the regulation of dauer formation by directly affecting the levels of this phosphatidylinositol.

***daf-18* Encodes the *C. elegans PTEN* Homolog.** A search of A *Caenorhabditis elegans* Database (28) found that *daf-18* (20), a known suppressor of *daf-2* IRL and *age-1* PI3′K mutations (21, 29, 30), maps close to the *PTEN* homolog. In addition to being dauer-formation defective, the only previously identified allele of *daf-18*, *e1375*, has several secondary phenotypes similar to those observed in the *nr2037* animals (21, 29, 30). We showed that *daf-18* corresponds to the *C. elegans PTEN* homolog by using four criteria.

First, we directly compared the vulval-bursting phenotype of *daf-18(e1375)* (30) and *nr2037* animals, because it is a phenotype that has not been reported for other dauer-formation

mutants (26). The *daf-18(e1375)* and *nr2037* mutant animals were found to have a similar incidence of vulval bursting (Table 1). Animals from both strains also had a predisposition to form bags of worms, and a small proportion of mutants from each strain was sterile or defective in laying eggs (ref. 21 and data not shown).

Second, we tested whether a genomic DNA fragment encoding the *C. elegans PTEN* homolog could rescue the defects of the *daf-18(e1375)* mutant allele. For ease of scoring, this experiment was conducted with a *daf-2(e1370);daf-18(e1375)* double mutant. As shown in Table 3, an 8-kb subclone of cosmid T07A9, in which the *PTEN* homolog is the only complete ORF, was sufficient to suppress the *daf-18(e1375)* phenotype and restore the temperature-sensitive dauer-constitutive phenotype of the *daf-2(e1370)* allele in four independent transgenic lines. The rescued animals maintained the dauer state at 25°C for at least 2 weeks, then recovered, and resumed development with kinetics similar to the *daf-2(e1370)* control when shifted to the permissive temperature (data not shown). We therefore conclude that the *C. elegans PTEN* homolog is sufficient to rescue the dauer-formation defects of the *daf-18* mutation.

Third, we showed that *daf-18(e1375)* and *nr2037* fail to complement one another in a dauer-formation test (Table 1). *daf-18(e1375)/nr2037* heterozygote animals formed very small numbers of dauers at a frequency similar to that of the *e1375* homozygotes (Table 1). Furthermore, both *daf-18(e1375)* and *nr2037* mutant animals show maternal rescue of the dauer-formation defects (see below).

Finally, sequence analysis of the *PTEN* locus in *daf-18(e1375)* animals showed that *e1375* consists of a complex series of tandem duplications in exon 4 of the *C. elegans PTEN* ORF (Fig. 2). The resulting alteration causes a frameshift that leads to a 6-aa insertion followed by a premature stop codon (Fig. 2). This change does not alter the sequences encoding the TAG/phosphatase domain but instead maps to the C-terminal portion of the *PTEN* ORF. No function has been ascribed to the C-terminal half of the PTEN protein but it is a frequent site of mutations in human tumors (1, 2, 5). These data prove that *daf-18(e1375)* and *nr2037* are allelic and that *daf-18* encodes the *C. elegans* homolog of the PTEN tumor suppressor.

Comparison of the Dauer-Epistasis and Maternal-Rescue Effects of the Two *daf-18* Alleles. We consistently found that the *daf-18(e1375)* animals were able to form dauers at low frequency under conditions in which the *daf-18(nr2037)* animals did not enter dauer diapause (Table 1). This finding, combined with the fact that the *daf-18(e1375)* mutation leaves the *C. elegans* PTEN phosphatase domain intact, suggested that the *e1375* allele may have residual PTEN activity. To address this issue, we compared the ability of the *daf-18(e1375)* and the *daf-18(nr2037)* alleles to suppress the phenotypes of *daf-2* IRL or *age-1* PI3′K loss-of-function mutations (Table 2). Consistent with previous studies (21, 29, 31), the *e1375* mutation poorly suppressed the constitutive dauer formation of *daf-2(e1370)* but largely rescued the dauer-constitutive phenotype of *age-1(m333)* (Table 2). The suppression of *age-1* PI3′K loss of function by the weak *daf-18(e1375)* allele was not complete, however, as these doubly mutant animals were somewhat developmentally delayed in comparison with *daf-18* single-mutant or wild-type nematodes (Table 2). In contrast, the *daf-18 PTEN nr2037* deletion allele completely suppressed the constitutive dauer-formation phenotypes of both *daf-2* and *age-1* mutants. This result suggests that the *e1375* allele retains residual PTEN function. Both alleles of *daf-18 PTEN* can suppress the decreased brood-size phenotype of *daf-2(e1370)* mutant animals (Table 2; 27, 28). Thus, fertility may be less sensitive to PtdIns-3,4,5-P_3 levels than dauer formation.

While constructing the double mutant animals used above, we noticed that both alleles of *daf-18 PTEN* showed a strong maternal rescue of the *daf-18* mutant dauer-formation defect.

Table 1. Vulval bursting and complementation tests

| Genotype | Animals that burst from vulva, % | n | Number of plates with indicated number of SDS-resistant dauers |
			0	1–2	3–10	>100
+/+	0	552	0	0	0	3
nr2037/nr2037	17.8	337	5	0	0	0
e1375/e1375	14.4	660	0	2	2	0
nr2037/e1375	ND	—	4	1	0	0

Adult hermaphrodites of a given phenotype were allowed to lay eggs for 4–6 h. Progeny were placed at 25°C and scored 2–4 days later. ND, not determined.

Table 2. Epistasis analysis with *daf*-2 IRL, *age*-1 PI3′K, and *daf*-18 PTEN

Daf-c genotype	Daf-d genotype	L4 larvae and adults, %	Dauers, %	Young larvae, %	*n*	Average brood size
+	+	99.3	0.0	0.7	876	233.6 ± 6.0
+	*daf-18(e1375)*	99.8*	0.0	0.2	1030	184.5 ± 17.3
+	*daf-18(nr2037)*	99.5*	0.0	0.5	943	211.7 ± 12.8
daf-2(e1370)	+	0.0	97.6	2.4	449	32.7 ± 10.6
daf-2(e1370)	*daf-18(e1375)*	1.2	98.8†	0.0	571	164.7 ± 22.6
daf-2(e1370)	*daf-18(nr2037)*	97.3*	0.0	2.7	754	222.2 ± 16.9
age-1(m333)‡	+	0.0	100.0	0.0	85	ND
age-1(m333)	*daf-18(e1375)*	99.6§	0.0	0.4	280	ND
age-1(m333)	*daf-18(nr2037)*	99.4*	0.0	0.6	360	ND
age-1(mg44)‡	+	0.0	97.4	0.6	79	ND
age-1(mg44)¶	*daf-18(nr2037)*	100.0	0.0	0.0	44	ND

For dauer counts, 1–10 adults per plate were allowed to lay eggs for 5 to 6 h. Progeny were scored 54–56 h after the eggs were laid. These data are the totals of at least two assays (generally two plates per assay) conducted on different days. *n* is the total number of progeny scored. See *Materials and Methods* for details regarding brood-size assays. Mean brood sizes are shown ±SEM. Experiments were performed at least twice with one representative data set shown. There were 4–10 parental hermaphrodites scored. ND, not determined.
*A few adults (0.6–1.5) had burst.
†These were "partial dauers." They were lighter and moved more spontaneously than true dauers; 99.2% grew into adults or L4 larva by 78 h after eggs were laid.
‡The adults that laid these eggs were the progeny of *age-1/mnC1* hermaphrodites (*mnC1* is a balancer for *age-1*). Plates were scored only if *mnCl* did not segregate; therefore, these are the progeny of *age-1* homozygotes.
§Of these, 46.9% were L4 larva. In contrast, for other genotypes that did not form dauers, the worms were overwhelmingly adults by this time.
¶These animals were only scored once.

Because *daf-18* loss of function suppresses *age-1(m333)*, 25% of the progeny of *age-1(m333);daf-18/+* hermaphrodites are predicted to be homozygous for the *daf-18* mutation and, therefore, to form normal adults rather than dauers. Instead, we found that nearly all the progeny of *age-1(m333);daf-18/+* hermaphrodites arrested as dauers. After 5 days, only 2.4% of these progeny grew to adults, and these were sterile (data not shown). This maternal rescue of *daf-18* function may explain why *daf-18* mutant alleles have not been identified in genetic screens for suppressors of *daf-2* IRL mutations, despite our finding that a strong loss-of-function *daf-18* allele can suppress *daf-2* IRL and *age-1* PI3′K loss-of-function mutations. A genetic screen for suppressors of *daf-2* IRL and *age-1* PI3′K mutations that takes into account the possibility of maternal rescue should yield other alleles of *daf-18* PTEN, as well as heretofore unidentified components of the *daf-2* IRL *age-1* PI3′K signaling pathway.

Table 3. Rescue of *daf-18(e1375)* by an 8-kb subclone of cosmid T07A9

Genotype	Rol animals, no.*		Non-rol, no.†	
	Dauers	Adults/L4	Dauers	Adult/L4
daf-2	—	—	134	0
daf-2; daf-18	—	—	3‡	392
daf-2; daf-18; array 1	21	0	0	16
daf-2; daf-18; array 2	37	0	5	21
daf-2; daf-18; array 3	33	0	1§	9
daf-2; daf-18; array 4	7	0	3	15

Adults hermaphrodites (*n* = 4–6) were allowed to lay eggs for 5–6 h. Progeny were placed at 25°C and scored visually 78 h after eggs were laid. These data are from one representative experiment. Experiments were performed at least twice.
*Transgenic animals were marked with the *rol-6* transgene.
†Non-roll animals are largely not transgenic, although a small proportion may be mosaic for the extrachromosomal array.
‡These were "partial dauers." They were lighter and moved more than true dauers.
§This animal looked like an L2d larva, because it was darker than a normal L2 larva.

DISCUSSION

DAF-18/PTEN Acts in the *C. elegans* PI3′K Signaling Pathway. We have identified a *C. elegans* homolog of the mammalian PTEN tumor suppressor and isolated animals that carry germ-line deletions within this gene. Based on four criteria, we conclude that the *C. elegans* PTEN homolog corresponds to a previously identified gene, *daf-18* (20): (*i*) the PTEN deletion mutant, *nr2037*, and the *daf-18(e1375)* alleles give rise to a common, diverse set of phenotypes; (*ii*) transformation with genomic DNA containing the *C. elegans* PTEN homolog can rescue the phenotypic effects of the *daf-18(e1375)* mutant allele; (*iii*) the *daf-18(e1375)* and *nr2037* alleles fail to complement one another for dauer formation; and (*iv*) *daf-18(e1375)* animals contain a mutant version of the *C. elegans* PTEN homolog that results in the premature termination of the ORF.

The *daf-18(nr2037)* deletion allele results in the loss of sequences encoding the PTEN phosphatase catalytic signature
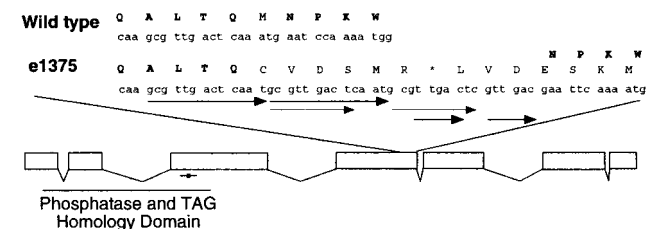

FIG. 2. *daf-18(e1375)* consists of a complex series of duplications in the *C. elegans* PTEN homolog. Standard one-letter codes for both DNA and amino acid sequences are shown, with the DNA sequences in lower case. *e1375* consists of a complex repeat structure in exon 4 of the *daf-18* PTEN gene. bp 4995 is deleted, followed by a 13-bp duplication of bp 4,983–4,995 (corresponding to the new mutant sequence; based on GenBank's numbering of T07A9). The first 10 bp of the repeat are duplicated again. This repeat is followed by a 6-bp subrepeat and then the insertion of an adenine. The complex repeat leads to a 6-aa insertion in exon 4, followed by a stop codon. The remainder of the exon is frameshifted with respect to the insertion. The active site of DAF-18 PTEN is indicated by an asterisk.

motif as well as much of the TAG homology domain. Accordingly, the *daf-18(nr2037)* deletion allele is likely a null mutation. Loss of the DAF-18 PTEN catalytic function abolishes dauer diapause. Epistasis analysis indicates that DAF-18 PTEN functions as a component of a PI3′K signaling pathway downstream of or parallel to DAF-2 IRL and AGE-1 PI3′K (Table 2; refs. 21 and 31). Because PtdIns-3,4,5-P_3 is both a product of PI3′K (14) and a substrate of PTEN (13), we propose that DAF-18 PTEN acts downstream of DAF-2 IRL to antagonize the activity of AGE-1 PI3′K (Fig. 3*a*).

The recruitment of the AGE-1 PI3′K by DAF-2 IRL should increase the levels of PtdIns-3,4,5-P_3. It is believed that this phosphatidylinositol and its derivatives positively regulate AKT-1 and AKT-2 (17), the *C. elegans* homologs of the mammalian Akt/PKB protooncogene (32), in a manner analogous to the activation of mammalian Akt (32). Once activated, AKT-1 and AKT-2 antagonize the function of the DAF-16 forkhead transcription factor, probably by direct phosphorylation (17). DAF-16 induces dauer-stage entry, and the inhibition of its activity by the AGE-1 PI3′K AKT signaling cascade is sufficient to prevent dauer formation (17). Based on our genetic studies and the known biochemical properties of PTEN, we propose that DAF-18 PTEN acts in opposition to PI3′K to reduce the levels of PtdIns-3,4,5-P_3, thereby downregulating AKT-1 and AKT-2 and restoring the activity of the DAF-16 forkhead transcription factor (Fig. 3*a*; refs. 17 and 18).

FIG. 3.   PI3′K signaling modules in mammals and nematodes have implications for cancer. (*a*) The *C. elegans* AGE-1 PI3′K homolog is recruited by the DAF-2 IRL protein. PI3′K generates PtdIns-3,4,5-P_3, whereas the DAF-18 PTEN homolog degrades this second messenger. PtdIns-3,4,5-P_3 and its derivative PtdIns-3,4-P_2 lead to the activation of Akt-family kinases. AKT-1 and AKT-2 may directly phosphorylate the DAF-16 forkhead transcription factor, thereby antagonizing its function (17). DAF-16 forkhead activity leads to induction of the dauer state (18, 19). Loss of DAF-16 activity prevents dauer formation. (*b*) In mammals, PTEN and PI3′K similarly antagonize one another to regulate local PtdIns-3,4,5-P_3 levels and thus Akt activation. Although *C. elegans* Akt homologs have not been shown to play a role in nematode programmed cell death, mammalian Akt can potently protect cells against apoptosis. DAF-16 homologs (18) of the FKHR family (38) may play a role in the regulation of apoptosis by Akt. (*c*) PTEN and PI3′K regulate the integration of extracellular survival signals into the normal cell division cycle. Loss of PTEN results in increased Akt activity and increased cell survival even in the presence of the proapoptotic cues induced by aberrant cell division.

In this manner, the relative levels and activities of DAF-18 PTEN and AGE-1 PI3′K will play a critical role in determining whether animals enter dauer diapause. Our data indicate that normal dauer formation depends absolutely on *daf-18* PTEN (Tables 1 and 2) and, therefore, presumably the downmodulation of PtdIns-3,4,5-P_3 levels.

The *daf-2* Signaling Pathway. Recent genetic studies have raised questions about the complexity of the DAF-2 IRL AGE-1 PI3′K pathway (17). Early work suggested that a single linear pathway exists downstream of DAF-2 IRL in which AGE-1 PI3′K acts to antagonize DAF-16 forkhead function (refs. 18 and 19; Fig. 3*a*). However, two published observations seem to be at odds with this model. First, both a gain-of-function *akt-1* mutation, *akt-1(mg144)*, and the weak *daf-18(e1375)* allele suppress *age-1*/PI3′K but not *daf-2*/IRL mutations (refs. 17, 21, 27, and 31; Table 2). Second, screens for *daf-2* IRL suppressors have yielded mutations within the *daf-16*-encoded transcription factor but not other components of the PI3′K pathway, such as *daf-18* PTEN (26). To explain the first observation, Paradis and Ruvkun (17) have proposed that DAF-2 IRL signaling may be transduced to the DAF-16 transcription factor by a second pathway that acts in parallel to PI3′K. Our findings suggest alternative explanations for these two observations that are consistent with the existence of a single DAF-2-dependent pathway.

The ability of *akt-1(mg144)* and *daf-18(e1375)* to suppress *age-1* PI3′K but not *daf-2* IRL mutations probably is due to the fact that many of these alleles result in only partial loss or gain of function. We have shown that *daf-18(e1375)* contains residual activity and that a strong loss-of-function *daf-18* PTEN allele can suppress both *age-1* PI3′K and *daf-2* IRL. The *akt(mg144)* mutant also may have intermediate activity in a manner analogous to *daf-18(e1375)*; however, because *akt-1(mg144)* is a gain-of-function allele, its strength is difficult to quantitate. Furthermore, we suggest that the *age-1(m333)* and *age-1(mg44)* animals are not null with respect to the AGE-1 PI3′K product PtdIns-3,4,5-P_3. This conclusion is based on the understanding that DAF-18 PTEN degrades PtdIns-3,4,5-P_3 and that, therefore, *daf-18* PTEN loss must rescue the *age-1* PI3′K mutant phenotype by the stabilization of some preexisting pool of PtdIns-3,4,5-P_3.

The failure of *daf-2* IRL genetic suppressor screens to identify *daf-18 PTEN* mutant alleles may be explained by the strong maternal rescue that we have observed for the *daf-18* PTEN alleles. This effect would cause both the F_1 and F_2 progeny of *daf-2* IRL mutant animals exposed to mutagens to adopt the dauer state irrespective of the status of their *daf-18* PTEN loci. A *daf-2* IRL genetic suppressor screen that accounts for possible maternal rescue should yield *daf-18* mutant alleles and also may lead to the identification of proteins involved in the recruitment or regulation of DAF-18 PTEN function.

Based on our findings, we favor a model in which DAF-2 IRL acts via a single linear pathway in which AGE-1 PI3′K and DAF-18 PTEN regulate AKT-1 and AKT-2 activity and, thus, the induction of dauer-stage entry by DAF-16 (Fig. 3*a*; refs. 16 and 17). However, it is also possible to modify the dual-pathway model to incorporate our data. Given the ability of the *daf-18* null allele to suppress *daf-2* IRL, we propose that the activity of the second (non-PI3′K) pathway is unmasked only when there is an intermediate level of AGE-1 PI3′K pathway activity (17). Alternatively, DAF-18 could be acting as a component of both the PI3′K and the non-PI3′K pathways. Further exploration of this pathway is required to distinguish between these models.

***daf-18* PTEN May Function in Another Signaling Pathway Independent of the DAF 2 IRL Cascade.** Previously, it has been noted that many *daf-18(e1375)* animals burst from the vulva as adults (ref. 30; Table 1). We have shown that the independently isolated *daf-18(nr2037)* deletion animals share the same phenotype, indicating that it is a bona fide consequence of *daf-18* PTEN loss of function. This secondary phenotype is not modified by the presence of *daf-2* IRL or *age-1* PI3′K mutant alleles (ref. 30 and data not shown) and has not been reported
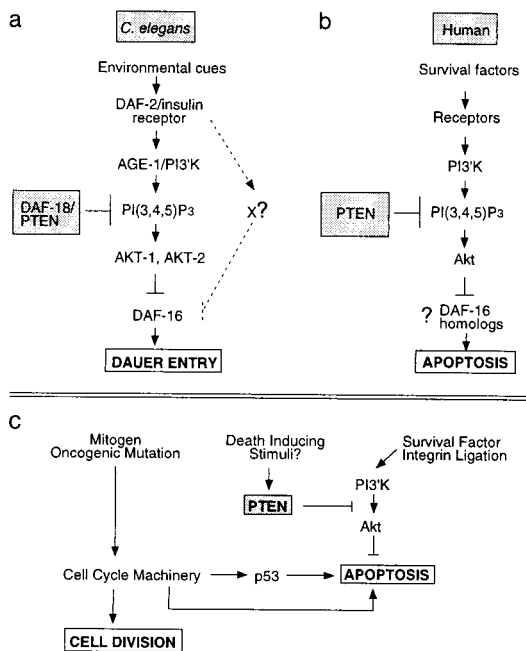
for animals carrying mutations in *daf-16*, the only other known dauer-formation-defective gene in the PI3′K pathway (26), suggesting that *daf-18* PTEN may be involved in a second signal transduction pathway that is independent of the *daf-2* IRL *age-1* PI3′K dauer-entry signaling cascade.

Implications for Cancer. The components involved in the PI3′K signaling module are conserved remarkably between humans and *C. elegans* with regard to order of action and structure (16, 17). Despite the conservation of this pathway, there are clear differences in the phenotypic output of Akt function. Although there is no evidence for the involvement of nematode AKT homologs in the regulation of programmed cell death, mammalian Akt can protect cells against a variety of apoptosis-inducing stimuli (32).

Many oncogenic mutations induce programmed cell death, and the outgrowth of malignancies depends on the inactivation of this program (Fig. 3*c*; ref. 33) This proapoptotic program can be circumvented by the up-regulation of survival cues, such as growth factor or integrin ligation (Fig. 3*c*). These survival cues are mediated frequently through the PI3′K/Akt pathway (32). Because Akt is regulated directly by PtdIns-3,4,5-P$_3$ levels (32), our results suggest that mammalian PTEN will function upstream of Akt to inhibit its death-protective activity (Fig. 3 *b* and *c*). Consistent with this idea, recent *in vitro* studies have shown that PTEN −/− mouse embryonic fibroblasts have elevated PtdIns-3,4,5-P$_3$, a concomitant increase in activated murine Akt, and heightened resistance to a variety of death-inducing stimuli (33).

It has been shown that a transforming growth factor β (TGF-β)-related signal transduction pathway (26) interacts with the *daf-2* IRL *age-1* PI3′K pathway to regulate entry into dauer diapause (17, 18, 26). It has been suggested that the convergence of the TGF-β and PI3′K signaling pathways may play a role in type II diabetes (17, 18, 34). We suggest that the TGF-β and PI3′K signaling pathways may also play a collaborative role in the suppression of human tumors. Intriguingly, loss of the TGF-β signaling component SMAD4 leads to juvenile polyposis coli (35), a disease phenocopied by loss-of-function mutations in PTEN (36, 37).

Implications for Type II Diabetes. The regulation of metabolism and longevity are linked in both *C. elegans* and mammals (22). Loss-of-function mutations in *daf-2* IRL or *age-1* PI3′K significantly increase the life span of mutant animals (22), and this increased longevity can be suppressed by *daf-18* PTEN mutation (29, 30). Both the *C. elegans* DAF-2 IRL molecule and the mammalian insulin receptor play roles in metabolic control. DAF-2 IRL signaling seems to be transduced largely by AGE-1 PI3′K (15, 16), and it has been proposed that factors in the corresponding human PI3′K pathway may be misregulated in type II diabetes (16, 18). Given the role of DAF-18 PTEN in the *C. elegans* DAF-2 insulin-like pathway, human PTEN may play a role in mammalian glucose homeostasis. Therefore, deregulation of human PTEN may predispose individuals to type II diabetes, and thus PTEN may be a rational pharmacological target for this disease.

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