DAF-9, a cytochrome P450 regulating *C. elegans* larval development and adult longevity

Kailiang Jia, Patrice S. Albert and Donald L. Riddle*

Molecular Biology Program and Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA
*Author for correspondence (e-mail: RiddleD@missouri.edu)

SUMMARY

The *daf-9* gene functions to integrate transforming growth factor-β and insulin-like signaling pathways to regulate *Caenorhabditis elegans* larval development. Mutations in *daf-9* result in transient dauer-like larval arrest, abnormal reproductive development, molting defects and increased adult longevity. The phenotype is sterol-dependent, and dependent on the activity of DAF-12, a nuclear hormone receptor. Genetic tests show that *daf-9* is upstream of *daf-12* in the genetic pathways for larval development and adult longevity. *daf-9* encodes a cytochrome P450 related to those involved in biosynthesis of steroid hormones in mammals.

We propose that it specifies a step in the biosynthetic pathway for a DAF-12 ligand, which might be a steroid. The surprising cellular specificity of *daf-9* expression (predominantly in two sensory neurons) supports a previously unrecognized role for these cells in neuroendocrine control of larval development, reproduction and life span.

Key words: Cytochrome P450, Dauer formation, Aging, *daf-9*, *daf-12*, TGF-β, Insulin

INTRODUCTION

C. elegans is a useful model for defining the signaling pathways that transduce environmental information into an appropriate behavioral/morphogenetic response by the developing organism (Riddle and Albert, 1997). When food is abundant, *C. elegans* develops directly to the reproductive adult through four larval stages, L1-L4, in three days. However, in an environment with limited food and high population density, larvae may arrest development at the second molt to enter the dauer stage (Cassada and Russell, 1975). The dauer larva is resistant to environmental stress, and it has a unique morphology, physiology and metabolism. It can live up to several months, greatly exceeding the normal life span of two weeks. It is considered to be non-aging because post-dauer life span is not affected by the duration of the dauer stage (Klass, 1976).

At least three environmental cues have been defined: food supply, temperature, and a constitutively secreted dauer-inducing pheromone that signals population density (Golden and Riddle, 1984a; Golden and Riddle, 1984b). Three functionally overlapping neural pathways control the developmental response to these cues. They involve TGF-β (Ren et al., 1996; Schackwitz et al., 1996), cyclic GMP (Birnby et al., 2000), and insulin-like (Kimura et al., 1997; Pierce et al., 2001) pathways, which relay the environmental signals to a nuclear receptor, DAF-12 (Antebi et al., 2000), to control dauer versus non-dauer morphogenesis. DAF-7, a TGF-β family member expressed in the ASI chemosensory neurons,

signals through DAF-4 type II (Estevez et al., 1993) and DAF-1 type I (Georgi et al., 1990; Gunther et al., 2000) transmembrane receptor kinases. These receptors control the activities of SMAD transcription factors encoded by *daf-8* (Estevez, 1997), *daf-14* (Inoue and Thomas, 2000) and *daf-3* (Patterson et al., 1997). DAF-2 (insulin/IGF receptor) is likely to signal to AGE-1, a PI(3) kinase catalytic subunit (Morris et al., 1996). This inhibits the activity of DAF-16, a member of the Forkhead family of transcription factors (Lin et al., 1997; Ogg et al., 1997). DAF-16 has been suggested to interact with DAF-3, DAF-8 and DAF-14 SMAD proteins to integrate DAF-7 and DAF-2 signals.

The *daf-2* insulin signal cascade also controls adult life span. Temperature-sensitive *daf-2* mutants raised at low temperature grow to adults with extended life spans (Kenyon et al., 1993; Larsen et al., 1995; Gems et al., 1998). *daf-16* activity is required for both dauer larva formation and the enhanced longevity of *daf-2* and *age-1* mutants.

The neuroendocrine mechanisms by which sensory transduction pathways are integrated to direct appropriate organism-wide physiological responses are poorly understood in any animal, and the functional relationship between DAF-12 and the Forkhead and SMAD family transcription factors in *C. elegans* has not been demonstrated by genetic analysis. The DAF-12 nuclear hormone receptor (Antebi et al., 2000) is involved in both dauer formation and adult life span. The *daf-12(m20)* mutation greatly enhances the extended life span of certain *daf-2* mutants (Larsen et al., 1995; Gems et al., 1998). Signals from the reproductive system require DAF-2, DAF-16

and DAF-12 activity to modulate *C. elegans* life span (Hsin and Kenyon, 1999). DAF-7 (TGF-β), DAF-2 (insulin) and DAF-11 (cGMP) signaling pathways may all be integrated by DAF-12.

DAF-12 is an orphan receptor because its ligand is unknown. Genetic results with *daf-9*, however, suggest that the neural signaling pathways modulate the level of a hormone that regulates DAF-12 activity. This hormone may be a key element in a neuroendocrine loop that links sensory information with reversible developmental/physiological responses in tissues throughout the body. Steroid hormone synthesis is catalyzed by members of the cytochrome P450 superfamily and by members of the steroid dehydrogenase family (Miller, 1988).

We report that *daf-9* encodes a cytochrome P450 that is expressed primarily in two sensory neurons. *daf-9* mutants have a pleiotropic phenotype including constitutive dauer-like arrest, abnormal reproductive development, molting defects, and increased adult longevity. Based on its sequence and on the sterol sensitivity of the mutant phenotype, we propose that DAF-9 is used to synthesize a DAF-12 ligand, possibly a steroid. Genetically upstream of *daf-12* function, *daf-9* defines a point of integration of the TGF-β and insulin signaling pathways.

MATERIALS AND METHODS

Genetics

Double mutants were constructed for genetic epistasis tests. *daf-12(m20)*, which exhibits a mild Lon phenotype, was used to construct a *daf-9 daf-12* double mutant. First, *unc-6 daf-12* males were crossed to *daf-9(e1406)/szT1* hermaphrodites. The cross-progeny that did not segregate Lon males were kept, and Lon non-Unc recombinants (*daf-9 + daf-12/+ unc-6 daf-12*) were picked. The *daf-9 daf-12* segregant is fertile and maintained as a homozygous stock. The strain *daf-16(m26); daf-9(e1406) +/+ unc-6(e78)* was constructed by mating *daf-16; unc-6/0* males with *unc-29(e1072); daf-9 +/+ unc-6* hermaphrodites. The cross-progeny were selfed and the desired strain isolated. The *daf-3 daf-9* animals were segregants of *daf-3(e1376) + daf-9(e1406)/daf-3(e1376) lon-2(e678) +*.

Life span

Dauer-like segregants (either *daf-9* homozygotes or *daf-9; daf-d* double mutants) were shifted from 20°C to 15°C 1-2 days after dauer arrest. Post-dauer adults, for life span measurements, were picked 5-7 days after entering the dauer-like stage. The *daf-16; daf-9* and *daf-3 daf-9* life spans were measured at 15°C, whereas the *daf-9* single mutants were tested at 25°C, 20°C and 15°C. Homozygous *daf-9(m540)* and *daf-9(e1406) daf-12(m20)* L4 hermaphrodites grown at 20°C were transferred to 25°C, 20°C and 15°C for survival tests. N2 and *daf-d* controls were treated similarly. All Tc1 insertion alleles were backcrossed up to eight times with N2 or *dpy-13* to remove other mutations that might affect the phenotype. SPSS Windows Version 10.0 was used for data analysis.

daf-9 cloning

Polymorphic sequence-tagged site mapping (Williams et al., 1992) was used to localize *daf-9*. For cosmid rescue, cosmid T13C5 DNA (100 ng/μl) and the pRF4 *rol-6(su1006)* marker plasmid (100 ng/μl) were microinjected into the ovaries of *daf-9(m540)* adults. Roller lines were scored for rescue as indicated by suppression of the Daf-c phenotype. The *rol-6* marker alone (100 ng/μl) was used to establish control transgenic lines.

A full-length *daf-9* cDNA was isolated by RT-PCR of Poly(A)+ mRNA from mixed-stage cultures. The 5′ part of the cDNA was amplified using 9-START/XbaI (5′-GCTCTAGAATGCCACT-CGTAATTGGCC-3′) and 9-EQLD (5′-ACGCGTCGACCC-TGCTTAGACTCTTGAAGGTC-3′) and cloned into the *Xba*I and *Sal*I sites of pBluescript/SK(+) (Stratagene). The 3′ part of the cDNA was amplified using 9-GDFM (5′-GGCATCAAGGTGATTTTATG-3′) and dT17 (5′-AACTGCAGGATCCTCGAGTTTTTTTTTTTTT-TTTTT-3′) in the first round, and reamplified using 9-GDFM and the dT17 adapter (5′-AACTGCAGGATCCTCGAG-3′). The PCR product was cloned into the pGEM-T vector (Promega) and then excised with *Nde*I and *Sal*I for ligation to the 5′ part of the cDNA. The full-length cDNA was verified by sequencing. SL1 (5′-TCTAGAATTCCGC-GGTTTAATTACCCAAGTTTG-3′) and 9-EXON5 (5′-AGTGGGT-CACGTAAGTCAATC-3′) primers were used to amplify the 5′ ends of the SL-1 *trans*-spliced products.

The cDNA rescue was performed and scored as described for the cosmid rescue except a *daf-9* full-length cDNA::*gfp* construct driven by 3 kb of *daf-9* 5′ sequence (100 ng/μl) (see *daf-9::gfp* below) was injected. A *gfp*-only construct driven by the same promoter, with *rol-6*, was used to establish control lines.

Sequencing mutants

To identify Tc1 transposon insertion sites, Tc1 primers with opposite orientations (OL7 and OL8) and *daf-9* gene-specific primers (Oxygen 5′ or Oxygen 3′) were used to amplify genomic DNA from *daf-9* strains *m540*, *m405/szT1*, *m641/szT1* and *m642/szT1*. The PCR fragments were cloned into pGEM-T and sequenced. For *m540*, Oxygen 3′ (5′-ATATTGAATAATGGTATATTTTTC-3′) was used with OL7 (5′-CCTTGTTCGAAGCCAGCTACAATGCC-3′) or OL8 (5′-TGATCGACTCGATGCCACGTCGTTGT-3′). For Tc1 insertion alleles *m405*, *m641* and *m642*, Oxygen 5′ (5′-TGTGATACCAC-CATGACAG-3′) and OL7 or OL8 were used. DNA from homozygous *daf-9(e1406)* animals was obtained from Daf-c larvae segregated from *e1406/szT1* and amplified using *daf-9* gene-specific primers. The PCR fragments were cloned into pGEM-T and sequenced.

Sterol supplementation

Normal NG agar plates (Brenner, 1974) with different concentrations of cholesterol, or 7-dehydrocholesterol, were seeded with *E. coli* strain OP50. Eggs from *daf-9(m540)* laid on the normal plates (5 μg/ml cholesterol) were transferred to normal and sterol-deficient plates, and development was examined at 20°C.

daf-9::gfp (green fluorescent protein)

The 3 kb *daf-9* promoter was amplified using 9P-5′ (5′-CTCG-CGCTTACTGCAGATTA-3′) and 9P-3′ (5′-CGGGATCCACTGT-ATTTTTTTTCGTCGCCA-3′). The PCR fragment was digested and cloned into the *Pst*I/*Bam*HI sites of *gfp* vectors pPD95.67 (with nuclear localization signal) and pPD95.75 (without nuclear localization signal). The full-length *daf-9* cDNA was amplified using 9-START/*Bam*HI (5′-CGGGATCCATGCCACTCGTAATTGGCC-3′) and 9-BalI (5′-CGCTGGCCAGTTGATGAGACGATTTCCGA-3′) and cloned into the *Bam*HI/*Bal*I site of *daf-9p::gfp* in pPD95.75.

To identify the GFP-expressing neurons in the head, we stained *daf-9* transgenic L1 animals using a DIO/calcium acetate protocol that stains IL2 neurons (C. Bargmann, personal communication), and we found GFP expression in two cells just posterior to the ventral DIO-stained IL2s. Two types of neurons are at this position: ventral IL1 neurons and URA neurons (White et al., 1986). However, their positions relative to IL2 differ from each other in the adult (White et al., 1986). Comparison of GFP-expressing cells in the L1 and adult showed that they likely corresponded to IL1, but since the positions of these cells are variable, the possibility that they are URA neurons could not be eliminated.

Cell killing

Laser microsurgery was performed on L1 larvae as described previously (Bargmann and Avery, 1995).

RESULTS

Pleiotropic phenotype of *daf-9*

The most obvious Daf-9 trait is the unconditional dauer-like arrest (Albert and Riddle, 1988). In the case of four severe mutants (*e1406*, *m405*, *m641*, and *m642*) about 30%-40% of the arrested animals exit the dauer-like stage, molt and grow to sterile (or nearly sterile) adults after several days. One homozygous viable mutant, *m540*, also exhibits non-conditional dauer-like arrest, but resumes development to fertile adulthood after 1-2 days. The average brood size of ten *m540* hermaphrodites was 171±58(s.d.) at 20°C.

The second highly penetrant trait in the *daf-9* severe alleles is a gonadal cell migration defect, and the development of an abnormal vulva. Normally, tips of the gonad reflex dorsally (Fig. 1A), but the severe *daf-9* mutant adults exhibit misplaced ovaries that may extend anteriorly or posteriorly on the ventral side after a failure to reflex. Fig. 1D shows a *daf-9(e1406)* adult in which the gonadal distal tip cell has migrated anteriorly to the pharynx. Fifty-seven *e1406* adults were scored for germ cell differentiation using Nomarski DIC optics. Oocytes were observed in 41, but sperm were not observed. Embryos and internally hatched larvae were found in four adults, indicating the production of sperm in at least a few animals. In normal animals, the vulva is a slightly protruding duct serving as the passageway through which fertilized eggs leave the uterus (Fig. 1B). All *daf-9* adults were either vulvaless or showed abnormal pseudovulval protrusions, and vesicles filled the uterine area (Fig. 1C). Other adult abnormalities include slow pharyngeal pumping, at about half the wild-type rate.

A third Daf-9 trait is a post-dauer molting defect. About 10% of the animals cannot properly shed the old cuticle after molting, which can result in death. Fig. 1E shows an adult that has shed the anterior portion of the post-dauer cuticle, which remained attached to the body.

The fourth Daf-9 trait is extended adult longevity. We measured the post-dauer adult life spans of the four severe *daf-9* mutants at 25°C, 20°C and 15°C (Fig. 2A). Maximum life span was substantially extended at 15°C, and modestly extended at 20°C and 25°C, whereas mean life span was increased only at 15°C, presumably because of deleterious aspects of the adult phenotype at higher temperatures. At 15°C, mean life spans were increased by 15-75%, whereas survival of the last quartile was increased 28-96% (Table 1). No increase in longevity was seen for the weak allele *m540* (Fig. 2A; Table 1).

Position of *daf-9* in the genetic pathway

Previously, *daf-9* was shown to function downstream of *daf-16* and *daf-5* in the genetic pathway for dauer formation (Albert and Riddle, 1988). To extend these data, we tested the interactions between *daf-9 X* and two X-linked dauer-defective (*daf-d*) genes in this pathway, *daf-3* and *daf-12* (see Materials and Methods), and the previously reported interaction between *daf-16* and *daf-9* was tested again. In tests of *daf-3* and *daf-16*, Daf-9 dauer-like larvae were segregated from *daf-d/daf-d*; *daf-9/+* parents in the ratio predicted if *daf-9* were fully epistatic to *daf-d* (Table 2). These data show that the *daf-9* mutation is epistatic to both *daf-3* and *daf-16*. By contrast, the *daf-9(e1406) daf-12(m20)* double mutant was Daf-d. It did not form any dauer-like larvae (Table 2), indicating that *daf-9(e1406)* acts upstream of *daf-12*. The *daf-12* mutation also
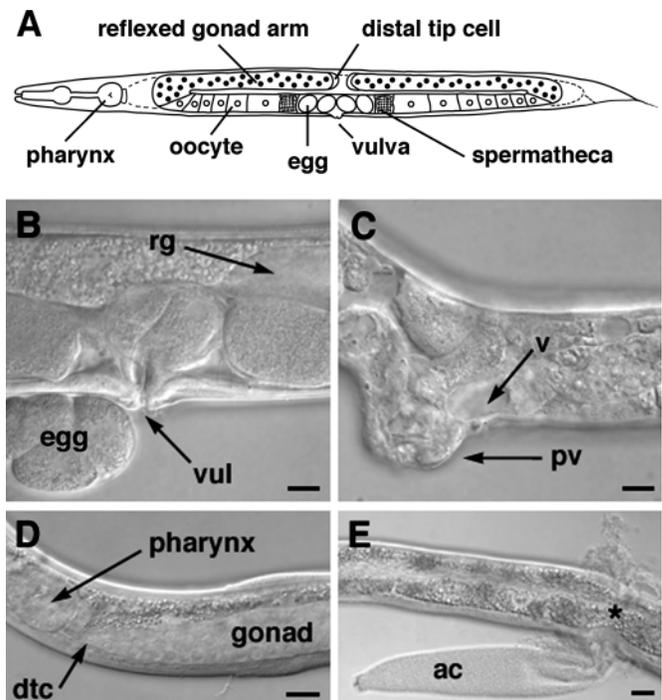

Fig. 1. *daf-9* mutant phenotype. (A) *C. elegans* hermaphrodite reproductive system. The gonad consists of anterior and posterior U-shaped tubular arms, with ovaries connecting to ventral spermathecae, which then join to the uterus, terminating at the vulva. (B) Normal morphology in a *daf-9(e1406) daf-12(m20)* double mutant. (C) Abnormal reproductive system of a *daf-9(e1406)* adult. Vesicles are present in much of the space normally occupied by the uterus. A large pseudovulval protrusion is shown. (D) Abnormal distal tip cell migration in *daf-9(e1406)*. The anterior gonad arm did not reflex dorsally, but extended anteriorly to the pharynx. (E) Molting defect of *daf-9(e1406)*. A shed anterior cuticle remains attached to the body, resulting in a constriction. rg, dorsal ovary of the reflexed gonad; vul, vulva; pv, pseudovulval protrusion; dtc, distal tip cell; v, vesicle; ac, shed anterior cuticle; ∗, constriction. Scale bars: 10 μm.

fully suppressed the abnormal reproductive development (Fig. 1B) and the molting defect. The *daf-9 daf-12* double mutant's brood size (274±32 s.d.; *n*=2735) was similar to *daf-12* and N2. Thus, *daf-12* activity is required for expression of the Daf-9 phenotype. We conclude that *daf-9* functions downstream of, or in parallel with *daf-3, daf-5* and *daf-16*, but upstream of *daf-12* in the genetic pathway for larval development.

We also tested the effect of *daf-3(e1376)*, *daf-12(m20)* and *daf-16(m26)*, which are not long-lived, on Daf-9 longevity at 15°C (Fig. 2B). The *daf-16* mutation, which suppresses the longevity phenotypes of *daf-2* and *age-1*, failed to suppress the longevity of *daf-9(e1406)* in two independent trials. The mean and maximum life spans were not significantly reduced in either trial (Fig. 2B; Table 1). *daf-3(e1376)* has a wild-type life span, but it greatly enhanced the life span of *daf-9(e1406)* mutants. The mean life span of the double mutant was increased by 80% (*P*<0.0001), and the survival of the last quartile was increased by 88% (Table 1).

The *daf-12(m20)* mutation was found to suppress the extended longevity of *daf-9(e1406)* (Fig. 2B; Table 1). The *daf-9(e1406) daf-12(m20)* double mutants had wild-type life spans

Table 1. Effect of temperature and genetic background on *daf-9* life span

Strain	Mean ± s.e.m.* (entire population)	Mean ± s.e.m.* (last quartile)	N†	P‡
daf-9 (25°C)				
N2	14.5±0.3	17±0.2	85, 41	
e1406	15.1±0.6	19±0.9	72, 30	0.055
m405	14.2±0.5	16±0.6	83, 45	0.901
m641	13.9±0.6	16±0.6	35, 59	0.350
m642	14.1±0.5	17±0.7	75, 11	0.789
daf-9 (20°C)				
N2	23.1±0.6	27±0.5	66, 24	
e1406	21.4±0.8	27±1.1	62, 32	0.905
m405	19.6±0.7	23±0.8	89, 31	0.013
m641	20.4±0.7	25±0.7	72, 20	0.028
m642	19.6±0.8	25±1.1	75, 9	0.027
daf-9 (15°C)				
N2	20.4±0.4	25±0.4	97, 51	
e1406	31.4±1.2	38±0.9	56, 35	<0.0001
m405	35.4±2.4	49±3.4	35, 18	<0.0001
m641	31.9±1.9	37±1.3	48, 17	<0.0001
m642	23.5±0.9	32±1.2	81, 38	<0.0001
m540	20.6±0.4	24±0.4	92, 31	0.838
daf-3 daf-9 (15°C)				
N2	20.9±0.4	25±0.4	82, 109	
daf-9(e1406)	27.7±1.2	38±1.1	44, 51	<0.0001
daf-3(e1376)	22.3±0.5	28±0.5	94, 106	0.001
daf-3 daf-9	37.5±1.5	47±1.9	41, 45	<0.0001 <0.0001 (*daf-3*) <0.0001 (*daf-9*)
daf-16; daf-9 (15°C)				
N2	23.9±0.4	27±0.4	82, 98	
daf-9(e1406)	28.4±1.0	34±1.7	44, 60	<0.0001
daf-16(m26)	20.4±0.3	23±0.2	111, 95	<0.0001
daf-16; daf-9	26.9±0.6	31±0.7	114, 108	<0.0001 <0.0001 (*daf-16*) 0.369 (*daf-9*)
daf-9 daf-12 (15°C)				
N2	22.8±0.4	26±0.2	82, 51	
daf-9(e1406)	33.8±1.2	41±2.1	44, 35	<0.0001
daf-12(m20)	22.8±0.5	27±0.3	100, 37	0.596
daf-12; daf-9	23.9±0.5	29±0.6	105, 39	0.005 0.007 (*daf-12*) <0.0001 (*daf-9*)

*s.e.m., standard error of mean.
†Two independent trials were pooled; population size for each trial is shown.
‡P values for mean life span of the entire population compared to N2 unless indicated in parentheses.

Table 2. Epistasis tests between *daf-9* and *daf-d* mutants

Strain	Progeny (%)*			Ratio (n†)
daf-16; daf-9 +/+ unc-6	Unc (25.2)	WT (53.4)	Dauer-like (21.3)	Unc: WT: Dauer=1:2.1:0.8 (1638)
daf-3+daf-9/daf-3 lon-2+	Lon (26.8)	WT (47.5)	Dauer-like (25.7)	Lon: WT: Dauer=1:1.8:1 (1176)
daf-9 daf-12		WT (100)	Dauer-like (0)	N/A‡ (2735)

*Percentage of total animals scored; †total number of animals scored; ‡not applicable.

at 25°C, 20°C and 15°C. Taken together, the genetic evidence suggests that *daf-9* functions downstream of the SMAD and Forkhead transcription factors, providing a point of integration for the branches of the dauer signaling pathway upstream of the *daf-12* nuclear receptor.

Molecular cloning of *daf-9*

A positional cloning strategy was used to identify *daf-9*, which had been mapped on the X chromosome between *lon-2* and *dpy-7* (Albert and Riddle, 1988). *daf-9* was found to lie between the physical markers *pKP943* and *stP33* (Fig. 3A). Cosmid T13C5 was found to fully rescue the *daf-9(m540)* Daf-c phenotype, i.e. the transgenic animals did not form dauer-like larvae, but grew to fertile adults. A 3.6 kb *Xba*I-*Sac*I genomic fragment from one candidate gene (T13C5.1) detected a Tc1 transposon insertion in each of the four *daf-9* Tc1 insertion
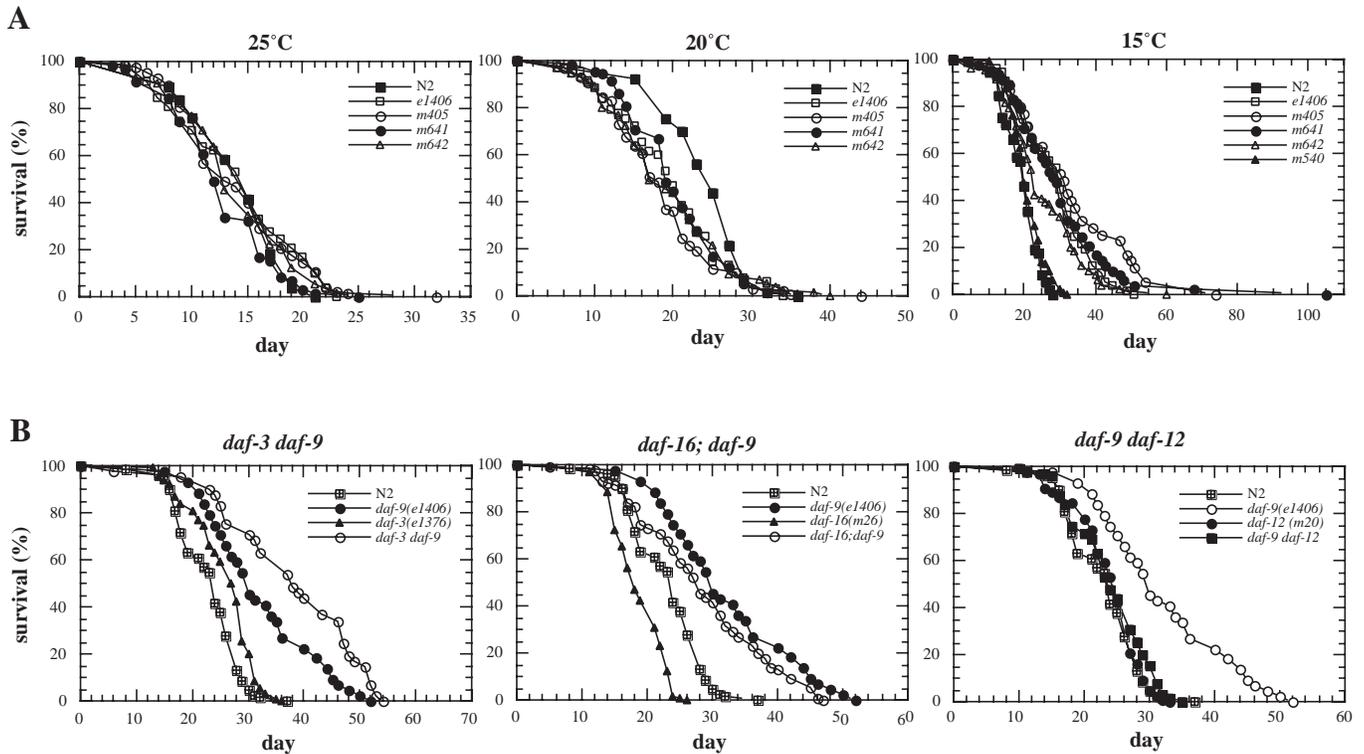
A

B

Fig. 2. Survival of wild type (N2), *daf-9* mutants and *daf-d; daf-9* double mutants. Each curve depicts one of the two independent trials represented in Table 1. The life spans were determined from the percentage of worms alive on a given day. (A) *daf-9* single mutants at three temperatures. (B) *daf-d; daf-9* double mutants at 15°C.

mutants (*m405*, *m540*, *m641* and *m642*) when used to probe Southern blots of mutant genomic DNAs (data not shown).

To further determine if T13C5.1 is *daf-9*, a full-length cDNA was isolated using RT-PCR. When introduced into the germline of *daf-9*(*m540*) under the control of a 3 kb *daf-9* promoter region, this cDNA completely complemented the Daf-c phenotype (Table 3). The sequence of the rescuing cDNA confirmed the gene structure predicted from the genomic sequence, with the exception of the first exon-intron boundary (see below). Fig. 3B shows gene and transcript structures and the mutation sites. A frameshift mutation in exon 2 was identified in the ethylmethane sulfonate (EMS)-induced reference allele, *e1406*. The first base of the CTC codon (L118) was deleted, a mutation predicted to result in a truncated, 172-amino-acid protein. A Tc1 transposon is inserted in the second intron of *m540* at the splice consensus site two nucleotides from the exon 2/intron 2 boundary. Tc1 insertions in exon 8 are predicted to truncate the protein in the severe alleles *m405*, *m641* and *m642*.

daf-9 encodes a cytochrome P450

The *daf-9* gene encodes a conceptual protein of 557 amino acids with a predicted molecular weight of 64.7 kDa. BLAST search results indicate that it is a cytochrome P450 hydroxylase. The cytochrome P450 family consists of heme-containing mono-oxygenases, which are involved in detoxification of xenobiotic compounds and synthesis and degradation of physiologically important molecules such as steroid hormones. Phylogenetic analysis showed that DAF-9 is most similar to members of the CYP2 family (e.g., rabbit 2C3,

a progesterone 16α-hydroxylase), and to steroid hydroxylases *Drosophila* CYP18 and human CYP17 and CYP21 (Nelson, 1998).

The steroid hydroxylases contain three conserved functional domains: the oxygen-binding domain (Ono sequence), the steroid-binding domain (Ozols tridecapeptide) and the heme-binding domain (Tremblay et al., 1994). DAF-9 shares 22-35% identity with CYP18, CYP21 and CYP17 in the steroid-binding domain; 56%, 56% and 38% identity, respectively, in the oxygen-binding domain; and 63-68% identity in the heme-binding domain. The highly conserved glycine and threonine in the oxygen-binding domain and the cysteine in the heme-binding domain are conserved in DAF-9 (Fig. 3C). The most closely related *C. elegans* protein is CYP23A1 (B0304.3), with 27% amino acid identity overall. The phylogenetic tree in Fig. 3D [adapted from Nelson (Nelson, 1998)] shows the relationships between DAF-9 and its homologs.

We have evidence for three alternatively spliced products based on RT-PCR results (Fig. 3B). Using a primer hybridizing at the ATG at the beginning of exon 1, an RT-PCR product containing exon 1 was amplified, but it encoded only the first 21 of the predicted 51 amino acids, indicating that the remaining 30-amino-acid predicted coding region is, in fact, part of intron 1. This RT-PCR product (isoform A), with a 63 nt exon 1, included all other exons spliced as predicted and it complemented the *daf-9* phenotype when placed under control of the *daf-9* promoter. Since most *C. elegans* transcripts are trans-spliced with SL1, we used an SL1 primer and a *daf-9* gene-specific primer for RT-PCR. We amplified two different products: SL1 trans-spliced to the second exon (isoform B) and
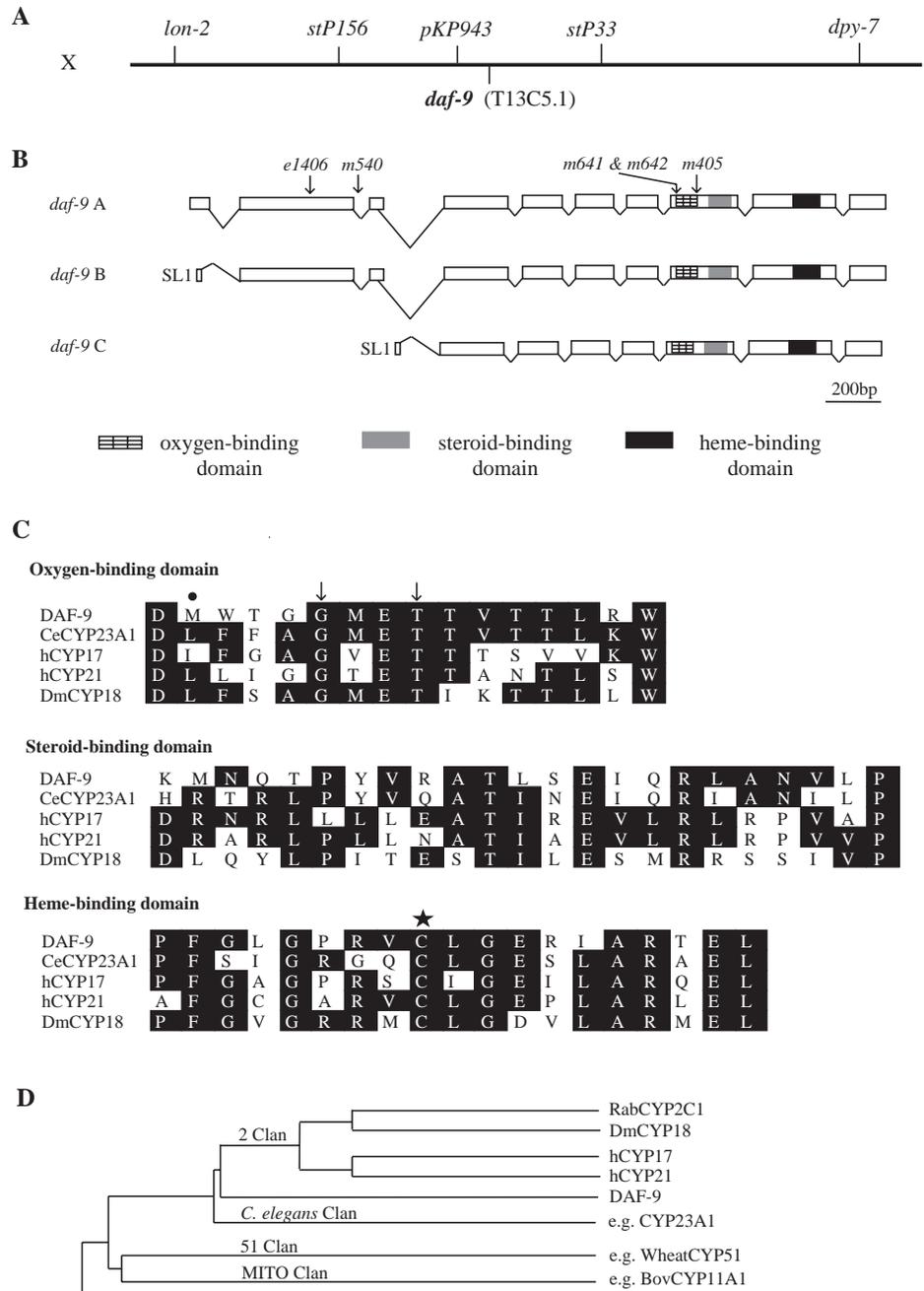
Fig. 3. *daf-9* encodes a cytochrome P450 hydroxylase. (A) Physical map of the *daf-9* region. *daf-9* (T13C5.1) is on cosmid T13C5. (B) Three forms of *daf-9* cDNA. Lines represent introns and boxes are exons. The predicted steroid-, oxygen- and heme-binding domains of DAF-9 are indicated. Sequence alterations for the five mutations indicated by arrows are described in Results. (C) Alignment of the three important functional domains of DAF-9 with its closest homologs: *C. elegans* CYP23A1, human CYP17, human CYP21 and *Drosophila* CYP18. Identical amino acids are in black boxes. The black dot in the oxygen-binding domain indicates the Tc1 insertion sites in m*641* and *m642* (inserted between the first and second bases in the codon for M356); the arrows indicate two amino acids important for oxygen binding, G360 and T363 (especially T363). The cysteine in the heme-binding domain, C496 (star), provides the proximal thiolate ligand for the heme. (D) Phylogenetic tree showing the relationship of DAF-9 and selected homologs (Nelson, 1998). In addition to the specific gene family members described in the text, rabbit CYP2C1 is included as a representative of the CYP2 family, bovine CYP11A1 represents the mitochondrial (MITO) clan, and wheat CYP51 represents the 51 clan. The GenBank accession number of the *daf-9* cDNA sequence is AF407572.

SL1 *trans*-spliced to the fourth exon (isoform C). The second exon starts with an ATG codon in the same reading frame as the first ATG in exon 1. Mutations *e1406* and *m540* affect isoforms A and B only, but the phenotype of *e1406* is not distinguishable from *m405, m641* and *m642,* which affect all three isoforms. Products B and C were not tested for *daf-9* activity.

The Daf-9 phenotype is modified by steroid supplementation

Since CYP21 and CYP17 are critical enzymes involved in the biosynthesis of steroid hormones in mammals, DAF-9 may have a similar function in *C. elegans*. If DAF-9 is involved in the biosynthesis of steroid hormones, decreasing the concentration of cholesterol, the precursor for steroids, should enhance the weak *daf-9(m540)* mutant phenotype, whereas providing steroids that bypass the requirement for DAF-9 function might reduce or eliminate the mutant phenotype.

We first examined the effect of reduced cholesterol on the Daf-c phenotype of *daf-9(m540)* at 20°C. On agar plates containing the level of cholesterol normally used in *C. elegans* growth medium (5 μg/ml), almost 100% of the *m540* larvae recovered by the second day after arrest at the dauer-like stage (Fig. 4A). However, when the cholesterol concentration was reduced, recovery was delayed and percent recovery reduced. Elimination of cholesterol reduced the final recovery percentage to roughly 20%, mimicking the phenotype of putative *daf-9* null alleles. The recovered *m540* adults were

Table 3. Rescue of *daf-9(m540)* by cloned *daf-9* full-length cDNA*

	Without *daf-9* cDNA[†]		With *daf-9* cDNA[‡]	
Strain	Background growth**	Strain	Rescue efficiency	
# 1	5.1% (1493)	# 5	100% (888)	
# 2	8.8% (505)	# 6	100% (1652)	
# 3	4.5% (1085)	# 7	100% (394)	
# 4	2.3% (1682)	# 8	100% (786)	

*Four independent transgenic lines were scored for each group; the number of transgenic animals scored for each strain is in parentheses.

[†]Representative genotype, *daf-9(m540) X; mEx117[rol-6(su1006) daf-9p::gfp]*.

[‡]Representative genotype, *daf-9(m540) X; mEx118[rol-6(su1006) daf-9p::daf-9cDNA::gfp]*.

**Whereas *daf-9(m540)* larvae very rarely developed directly to the adult, the transgenic lines exhibited a background of direct development.

very sick and most of them were sterile, like the null mutant. The observed cholesterol dependence suggests that the *daf-9(m540)* block is incomplete, and higher levels of the precursor cholesterol ameliorate the defect. Constitutively formed *daf-7(e1372)* dauer larvae were used as a control in this experiment and their recovery at 20°C was not cholesterol-dependent (Fig. 4B). Furthermore, the *daf-7* adults were neither sick nor sterile. These results indicate that DAF-9 is very likely involved in synthesis of a steroid that promotes larval growth and reproductive development, and this function is cholesterol dependent.

We tested the effect of other sterols on *daf-9(m540)* reproduction to find a possible substrate or product of DAF-9. We tested pregnenolone, the first intermediate in the pathway for mammalian steroid hormone biosynthesis; 7-dehydrocholesterol, the most abundant sterol in *C. elegans* (Chitwood, 1999); and the insect molting hormones, ecdysone and 20-hydroxyecdysone. 7-dehydrocholesterol can substitute for the function of cholesterol (Fig. 4C). However, the other sterols had no effect on wild type or on expression of the *daf-9(m540)* phenotype (data not shown).

Expression pattern of *daf-9*

If *daf-9* is a target of the dauer-inhibiting TGF-β and insulin-like signaling pathways, it is of interest to determine the cells in which it is expressed. We fused the *daf-9* full-length cDNA in frame with *gfp* coding sequence (Chalfie et al., 1994) under the control of the 3 kb *daf-9* promoter region. When injected into the germline of *daf-9(m540)*, it fully rescued the Daf-c phenotype (Table 3), indicating that the *gfp* reporter must be expressed in cells required for *daf-9* function. The DAF-9::GFP fusion protein was expressed mainly in a pair of neurons anterior to the nerve ring (Fig. 5), and the signal was detected in all stages from late embryos to adult. Expression in embryos was observed only in those which developed at 25°C. The intensity of the signal was not noticeably different in stages from L2 to adult, or in the dauer larva, but was fainter in embryos and L1 larvae. Weak expression was also observed in the spermathecae of older adults raised at 25°C.

We tentatively identified the two cells strongly expressing GFP as right and left ventral Inner Labial-1 (IL1) neurons, but could not exclude right and left ventral URA neurons. There are six IL1 neurons, which extend afferent processes anteriorly
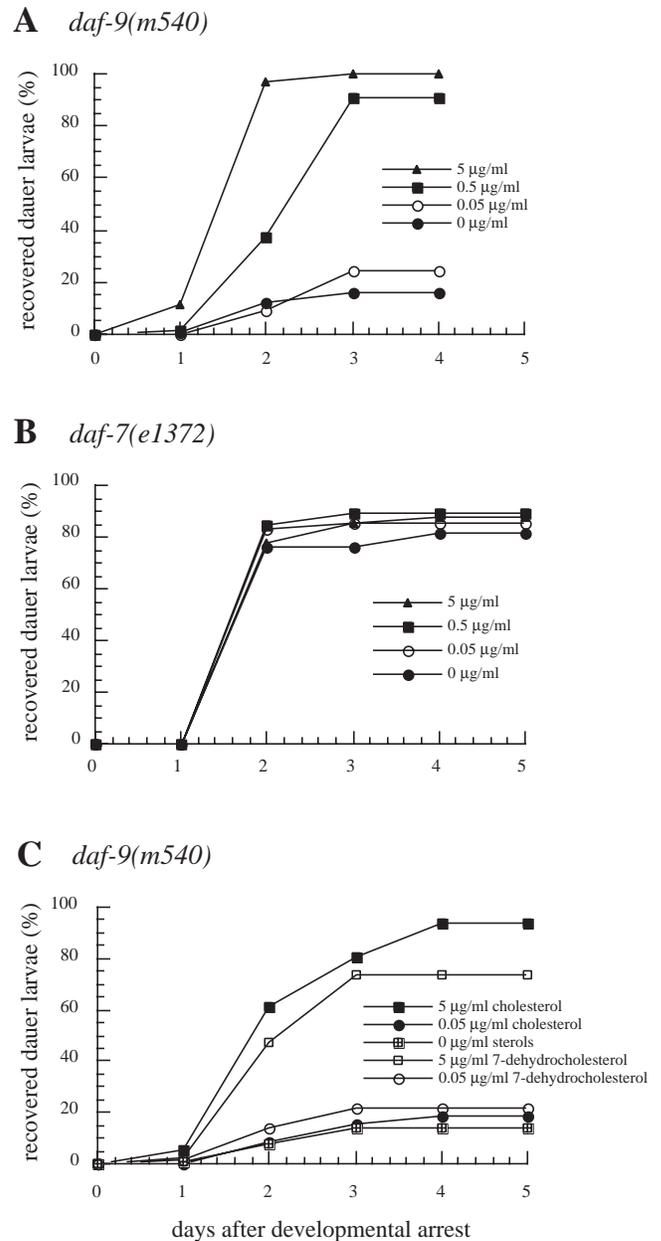
Fig. 4. The Daf-9 phenotype is sterol dependent. (A) DAF-9 is required for normal non-dauer development. Recovery of *daf-9(m540)* from dauer-like arrest was assayed at 20°C on NG agar plates with cholesterol concentrations as shown. Data are presented as percent recovery on a given day after dauer-like arrest. (B) Cholesterol limitation had no effect on the recovery of the *daf-7(e1372)* mutant, which is Daf-c due to reduced TGF-β signaling. (C) 7-dehydrocholesterol substituted for cholesterol to promote the recovery of *daf-9(m540)*.

from cell bodies behind the first pharyngeal bulb (metacorpus) to the anterior tip of the head (White et al., 1986). Their axons project posteriorly into the circumpharyngeal nerve ring. There are four URA motorneurons, or sensory motorneurons, that innervate muscles in the head via neuromuscular junctions in the nerve ring. Their cell bodies are also located behind the metacorpus and anterior to the nerve ring, and they extend anterior processes into four of the six labial sensory process
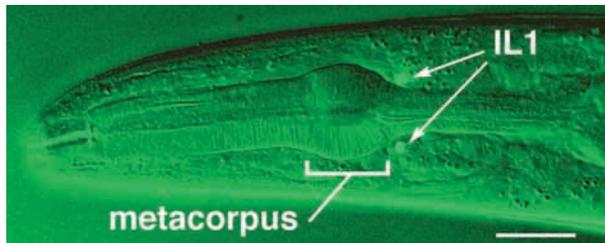
Fig. 5. Expression of *daf-9*. *daf-9p::daf-9cDNA::gfp* expression in the *daf-9(m540)* background. An intense reporter signal was confined to two cell bodies that are posterior to the metacorpus of the pharynx and anterior to the nerve ring. These two cells (arrows) were identified as right and left ventral IL1 neurons, or possibly ventral URA neurons (ventral view). Scale bar, 20 μm.

Fig. 6. Revised pathway for dauer formation and adult longevity. Proposed wild-type function is shown, with arrows indicating stimulation of activities and T-bars indicating inhibition, but the steps do not indicate direct protein interactions. "ON" or "OFF" activity states are given for conditions that promote dauer formation and adult longevity. *daf-2* and *daf-7* branches converge at *daf-9*. DAF-9 inhibits dauer formation and shortens life span by inactivating the DAF-12 nuclear receptor (NR). See Discussion for details.

bundles (White et al., 1986). In some transgenic animals, DAF-9::GFP was only expressed in one of the ventral cells. The positions of these two cell bodies are variable, with one occasionally found anterior to the metacorpus.

Two other *gfp* constructs were made, in which the 3 kb *daf-9* promoter drives the expression of *gfp* with or without a nuclear localization signal (see Materials and Methods). The expression of these transgenes in N2 and *m540* genetic backgrounds was the same. In addition to the pair of IL1/URA neurons, one to four other cells likely to be neurons posterior to the nerve ring occasionally gave faint signals. These latter cells were not seen in the DAF-9::GFP construct. The DAF-9::GFP fusion protein was very finely localized within the cell bodies (Fig. 5), whereas GFP driven by the same promoter, but without *daf-9* coding sequences, diffused throughout the cell body (not shown). This suggests that DAF-9 might be membrane localized. Membrane-bound cytochrome P450s have a hydrophobic anchor sequence at their N terminus. The hydropathy analysis of DAF-9 as predicted from genomic sequence did show such a region (residues 36-60).

The strong expression of DAF-9 in the ventral IL1/URA neurons suggests that they are involved in regulation of dauer formation. We tested this by destroying the two GFP-expressing neurons with a laser microbeam to see if the surgery mimics the Daf-c phenotypes. Early L1 larvae carrying *daf-9p::gfp* and *rol-6* in an N2 background were treated. Six successfully treated animals (as judged by loss of GFP fluorescence) from two independent experiments did not form dauer larvae constitutively. It is possible that the surgery was performed too late to produce an effect, or that neuronal function may be redundant with other cells that were not ablated.

To further test the possible functional link between DAF-9 and neuronal expression, we surgically treated *daf-9(m540)* L1 larvae that carried *daf-9p::daf-9cDNA::gfp* and *rol-6* transgenes. Four treated roller animals obtained from three independent experiments lost GFP expression, and all formed dauer-like larvae constitutively. Three of them recovered from dauer-like arrest in 1-2 days; two were sterile and one produced 42 progeny. The fourth recovered after 1 week of dauer-like arrest, and died of internal hatching with two progeny. Loss of the two ventral neurons restored the mutant *m540* Daf-c phenotype and induced adult sterility, which is characteristic of the severe alleles. Eight treated animals that retained GFP expression in at least one cell grew directly to fertile adults.
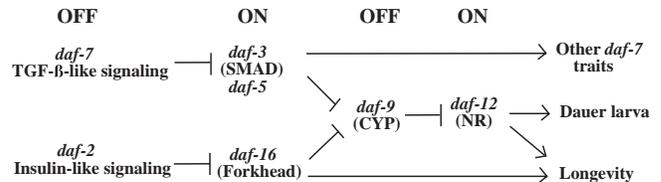
These data indicate that expression of the *daf-9* cDNA in the IL1/URA neurons is necessary for rescue of the Daf-9 phenotype in the transgenic line.

DISCUSSION

daf-9 encodes a cytochrome P450 hydroxylase

Mutations in *daf-9* result in a pleiotropic phenotype including dauer-like larval arrest, defects in gonadal cell migration, an abnormal vulva, molting defects and increased adult life span. Two pieces of evidence indicate that the gene we cloned, T13C5.1, is *daf-9*. First, the full-length cDNA rescued the mutant phenotype of *m540*. Secondly, mutations were identified in five independent *daf-9* mutants, and the mutant sequences were consistent with the severity of the mutant phenotypes. The Tc1 insertion in the second intron in *m540* results in a leaky phenotype, consistent with occasional somatic removal of Tc1 by RNA splicing (Rushforth and Anderson, 1996). Three Tc1 insertions in the oxygen-binding domain sequence are predicted to truncate the protein in the *m405*, *m641* and *m642* alleles. They are homozygous sterile, as is the EMS-induced allele, *e1406*, which carries a frameshift mutation in exon 2 resulting in a truncated polypeptide missing all three conserved domains. These four alleles are putative null alleles.

The predicted DAF-9 amino acid sequence indicates that it is a member of the cytochrome P450 hydroxylase family (Nelson, 1998; Gotoh, 1998) similar to the CYP2 family, and to human CYP17 and CYP21, *Drosophila* CYP18, and *C. elegans* CYP23A1. Based on sequence similarity to key hydroxylases (CYP17 and CYP21) involved in biosynthesis of steroid hormones in mammals, DAF-9 is hypothesized to catalyze a step in the pathway to synthesize steroid hormones in *C. elegans*.

Genetic pathway for dauer formation and adult longevity

A complex genetic pathway controls *C. elegans* dauer formation (Riddle and Albert, 1997). The genes in this pathway were ordered on the basis of genetic epistasis tests that determined whether *daf-c; daf-d* double mutants formed dauer larvae constitutively or grew to the adult at 25°C. The epistatic mutation is judged to block downstream in the pathway because its mutant phenotype is unaffected by perturbations in

upstream signaling. Since mutations in *daf-3*, *daf-5*, *daf-12* and *daf-16* are Daf-d, direct epistasis tests between these genes have not been performed. However, the *daf-12* gene was previously positioned in a branch of the pathway downstream of *daf-3* and *daf-5* because only *daf-3* and *daf-5* are required for expression of other aspects of the upstream Daf-c mutant phenotype, such as persistent display of an L1 surface antigen in all larval stages (Grenache et al., 1996). Prior to examination of *daf-9*, which is Daf-c, there were no direct genetic epistasis data to support this divergence.

The *daf-3*, *daf-5* and *daf-16* genes are positioned upstream of, or in parallel with, *daf-9* because they fail to suppress mutations in *daf-9*, whereas *daf-12* acts downstream of *daf-9* because the *daf-12* mutation is fully epistatic. Regulation of DAF-16 activity is the major output for DAF-2/insulin signaling (Kenyon et al., 1993; Larsen et al., 1995), and DAF-7/TGF-β signals through *daf-3* and *daf-5* (Riddle and Albert, 1997). Since *daf-9* functions downstream of all these genes, the *daf-7* and *daf-2* branches of the genetic pathways are judged to converge at *daf-9*. It is possible that *daf-9* could be bifunctional, acting in two parallel pathways leading to *daf-12*, but the simplest model is that there is a single pathway from *daf-9* to *daf-12*. *daf-12* activity is required for expression of all aspects of the Daf-9 phenotype.

Results with *daf-9* expand our knowledge about the signaling pathways that control *C. elegans* life span. To our knowledge, *daf-9* is the first mutant identified downstream of *daf-16* that extends adult longevity. DAF-9 activity is probably not the only output of *daf-16* in control of life span. It was proposed previously that *daf-16* has two outputs, only one of which is to function with *daf-12* (Gems et al., 1998). The Daf-c mutant adults in the *daf-7* (TGF-β) branch do not exhibit a longevity phenotype. However, King (King, 1998) observed that *daf-3* and *daf-5* mutations enhance *daf-2(e1370)* longevity. The life span of *daf-3 daf-9* suggested that *daf-3* could function through *daf-9* because the *daf-3* mutation enhances the post-dauer longevity of *daf-9(e1406)*. Since *daf-9* is one of the outputs of *daf-2*/*daf-16* branch, it is not surprising that *daf-3* and *daf-5* also interact with *daf-2* mutations. The interactions suggest that TGF-β signaling normally plays a larger role in larval development than in adult longevity, because the effect of *daf-3* and *daf-5* loss of function on longevity is only apparent in a background of reduced insulin signaling.

The data can be interpreted in the following integrated model (Fig. 6). In the case of dauer formation, the *daf-2* and *daf-7* branches both influence the production of the ligand for DAF-12 by affecting the activity of DAF-9. The wild-type *daf-9(+)* gene acts to inhibit dauer formation by inactivating DAF-12 dauer promoting activity. DAF-2 antagonizes the activity of DAF-16, and DAF-7 inhibits the function of DAF-3 and DAF-5. DAF-3, DAF-5 and DAF-16 function together to inhibit DAF-9 activity, perhaps by depriving it of a substrate. Loss of function of either the *daf-2* or *daf-7* pathway will inhibit *daf-9* gene activity, resulting in dauer arrest. For the control of adult life span, *daf-9(+)* shortens life by inhibiting DAF-12. In a *daf-2* mutant, DAF-16 is active and extends life span by inhibiting *daf-9* activity and activating/inhibiting the other longevity determining genes. This genetic model provides the first suggestion of the interactions between *daf-3* SMAD/*daf-16* Forkhead transcription factors and the DAF-12 nuclear receptor in controlling dauer formation, and reveals new information about the regulation of life span, at least at low temperature. It also gives clues about the function of *daf-12* in this pathway.

Mechanism by which *daf-9* regulates life span

Life span in *C. elegans* is influenced by at least four overlapping factors: caloric restriction, DAF-2/insulin-like signaling, gonadal signaling and the *clk* pathway. Mutations in *clk* genes slow the rates of many processes, including post-embryonic development and pharyngeal pumping (Lakowski and Hekimi, 1996). Certain *eat* mutants (which have insufficient food intake and a starved appearance due to pharyngeal dysfunction) are long-lived, and this is thought to result from caloric restriction. Genetic epistasis indicates that these mutations affect the same genetic pathway as *clk-1*, but different from *daf-2* (Lakowski and Hekimi, 1998). Since *daf-9* adults pump slowly, it is possible that *daf-9* longevity results from caloric restriction. However, *daf-9* mutants are not slowed in development from egg to L2, and neither *clk* nor *eat* genes are involved in dauer formation. *daf-9* appears to function independently of the *clk* pathway, as do other *daf* genes. DAF-12 activity is required for the increased longevity resulting from germline elimination (Hsin and Kenyon, 1999). We propose that *daf-9* also functions in this pathway. Spermathecal expression of the *daf-9* GFP reporter in adults is consistent with such a role.

DAF-9 and steroid biosynthesis

C. elegans requires dietary sterol for growth and reproduction, and it metabolizes sterols (Chitwood, 1999), but the function of these products is not understood. Reducing the concentration of cholesterol (the precursor for all steroid hormones) in agar plates greatly inhibited the post-dauer development of *daf-9(m540)*. We reason that limitation of cholesterol in the medium reduced the concentration of DAF-9 substrate, in turn causing a decrease of the DAF-9 product. The ability of the severe *daf-9* mutants to grow to sterile adults may result partially from redundant activity of a related protein, like CYP23A1.

DAF-9 may function in one of several steps to produce a hormone. The most abundant nematode sterol is 7-dehydrocholesterol, and metabolism of cholesterol to 7-dehydrocholesterol in *C. elegans* has been proposed (Chitwood, 1999). Our data show that 7-dehydrocholesterol was not significantly different from cholesterol itself in rescuing *daf-9(m540)*. Hence, 7-dehydrocholesterol may be in the pathway for hormone biosynthesis, but prior to the *daf-9* block. Alternatively, 7-dehydrocholesterol may be metabolized into cholesterol first, then to other sterols required for DAF-9 function.

The distribution and transport of sterols in *C. elegans* has recently been described by Matyash et al. (Matyash et al., 2001). At least two *C. elegans* mutants, other than *daf-9*, affected in sterol metabolism are sensitive to cholesterol limitation, including the steroid dehydrogenase mutant, *let-767* (L. Kuervers and D. Baillie, personal communication), and a homolog of human *NPC1* (Sym et al., 2000). *NPC1* encodes a protein implicated in the transport of sterols to lysosomes (Neufeld et al., 1999). In *C. elegans, npc-1 npc-2* double mutants form dauer larvae constitutively (Sym et al., 2000). These observations are consistent with our hypothesis that cholesterol is the precursor for the DAF-9 substrate.

DAF-9 may synthesize the ligand for DAF-12

The evidence that DAF-9 may be used to synthesize the ligand for DAF-12 can be summarized as follows. First, a *daf-12* mutation suppresses all aspects of the pleiotropic Daf-9 phenotype showing that *daf-12* function is required for expression of all *daf-9* traits. We conclude that *daf-9* normally acts through *daf-12*. Second, certain *daf-12* mutants resemble *daf-9* mutants. Rare recessive Daf-c mutations affect the ligand-binding domain of DAF-12, whereas Daf-d mutations affect the DNA-binding domain (Antebi et al., 2000). The null phenotype appears to be Daf-d. The *daf-9* mutant is Daf-c (similar in appearance to the Daf-c ligand-binding domain mutants of *daf-12*), consistent with a role for DAF-9 in synthesis of the ligand for DAF-12. Third, *daf-9* and *daf-12* mutants share a similar gonadal migration defect. One interpretation of these data is that the Daf-c mutations prevent ligand synthesis and ligand binding, respectively.

Usually, nuclear hormone receptors are activated by their ligands (Whitefield et al., 1999). The role of this putative ligand is to activate the growth-promoting function of DAF-12, or to inhibit the dauer-promoting function. DAF-12 appears to be involved in both processes, essential for dauer formation and redundant for growth (Antebi et al., 2000). It is also possible that the molecule made by DAF-9 is the ligand for another nuclear receptor, which is a transcriptional repressor and partner of DAF-12.

There are predicted to be about 250 nuclear receptor genes in the *C. elegans* genome (Sluder et al., 1999). Although *daf-12* mutations completely suppress the Daf-9 phenotype, suggesting that *daf-9* may function specifically to make a ligand for DAF-12, the possibility that DAF-9 is also involved in the production of ligands for other nuclear receptors cannot be excluded. *daf-9* mutations are pleiotropic, and more subtle traits (not suppressed by a *daf-12* mutation) may have been missed.

daf-9 expression in sensory neurons

The *daf-9* promoter is active primarily in two IL1/URA neurons (a bilateral pair on the ventral side of the head). These neurons have not been previously implicated in dauer formation or adult life span, but the IL1 neurons have been proposed to be mechanosensory cells involved in feeding behavior (Hart et al., 1995). It is possible that mechanosensory stimulation during feeding may increase DAF-9 activity to stimulate hormone production favoring non-dauer development.

Our *daf-9p::cDNA::gfp* reporter construct complements (rescues) the Daf-9 mutant phenotype, suggesting that GFP is expressed in all cells required for *daf-9* function. Destruction of these cells in the transgenic line conveyed the Daf-c and sterile adult mutant phenotypes, characteristic of the severe *daf-9* alleles. This result indicates a role for the two neurons in promoting larval development and adult fertility. However, killing these cells in a *daf-9*(+) genetic background did not result in a Daf-c phenocopy. Therefore, it is possible that *daf-9* is also normally expressed in other cells, and this expression is functionally redundant in wild type.

daf-2 functions in the nervous system to regulate dauer formation and life span (Apfeld and Kenyon, 1998; Wolkow et al., 2000), suggesting that the DAF-2/insulin pathway regulates the activity of *daf-9* through a neural network. The steroid hormone produced in IL1/URA neurons would act in a subsequent, as-yet-unknown step (or steps) and eventually be utilized by DAF-12, which is expressed in many tissues (Antebi et al., 2000), to control dauer formation. Neuronal expression of cytochrome P450s used to synthesize steroids has been documented in vertebrates (Kazuyoshi et al., 1999).

The *daf-9* promoter is active in all stages including the dauer stage, suggesting that DAF-9 activity may be regulated posttranscriptionally. One of the DAF-9 homologs in the CYP2 family, CYP2E1, has been reported to be posttranscriptionally regulated in rodents (Gonzalez et al., 1991). Consistent expression of the GFP fusion protein in all stages seems not to support this type of regulation for DAF-9. Wild-type DAF-9 function may be regulated by the availability of its substrate.

Conclusion

We recently described the functions of the *daf-12* nuclear receptor, an orphan receptor that acts downstream of the TGF-β and insulin signaling pathways controlling *C. elegans* dauer larva development (Antebi et al., 2000). The results described here build on that knowledge, identifying *daf-9* as a point of integration for the TGF-β and insulin signaling pathways just upstream of *daf-12*. We propose that *daf-9* is involved in synthesis of a ligand for the *daf-12* receptor. The phenotype of a weak *daf-9* mutant is enhanced by cholesterol limitation, implicating *daf-9* in sterol metabolism. It is the first of the 80 *C. elegans* cytochrome P450 genes (The *C. elegans* Sequencing Consortium, 1998; Gotoh, 1998) to be associated with a specific biological function. The surprising cellular specificity of *daf-9* expression (predominantly in two neurons) supports a previously unrecognized role for these cells in control of dauer formation and adult fecundity.

The results suggest that the mechanism for coordination of diapause, reproductive development and adult longevity may be steroid hormone regulation, and they provide a neuroendocrine link from upstream insulin and TGF-β signaling pathways to nuclear hormone receptor activity. This link may be relevant to the understanding of such pathways in mammals.

Note added in proof

Similar results on *daf-9* have been reported by Gerisch et al. (Gerisch et al., 2002).

REFERENCES

Albert, P. S. and Riddle, D. L. (1988). Mutants of *Caenorhabditis elegans* that form dauer-like larvae. *Dev. Biol.* **126**, 270-293.

Antebi, A., Yeh, W-H., Tait, D., Hedgecock, E. and Riddle, D. L. (2000). *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes Dev.* **14**, 1512-1527.

Apfeld, A. and Kenyon, C. (1998). Cell nonautonomy of *C. elegans daf-2* function in the regulation of diapause and life span. *Cell* **95**, 199-210.

Bargmann, C. and Avery, L. (1995). Laser killing of cells in *Caenorhabditis elegans*. In *Methods in Cell Biology*. Vol. 48 *Caenorhabditis elegans*: *Modern Biological Analysis of an Organism* (ed. H. F. Epstein and D. C. Shakes), pp. 225-250. San Diego: Academic Press.

Birnby, D. A., Malone, E., Vowels, J. J., Tian, H., Colacurcio, P. L. and Thomas, J. H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. *Genetics* **155**, 85-104.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

The *C. elegans* Sequencing Consortium (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012-2018.

Cassada, R. C. and Russell, R. L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **46**, 326-342.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Chitwood, D. J. (1999). Biochemistry and function of nematode steroids. *Biochem. Mol. Biol.* **34**, 273-284.

Estevez, A. O. Z. (1997). *The Role of the* daf-8 *Gene in* Caenorhabditis elegans *Dauer Larva Development*. Ph. D. Thesis. Division of Biological Sciences, University of Missouri, Columbia.

Estevez, M., Attisano, L., Wrana, J. L., Albert, P. S., Massague, J. and Riddle, D. L. (1993). The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* **365**, 644-649.

Gems, D., Sutton, A. J., Sundermeyer, M. L., Albert, P. S., King, K. V., Edgley, M. L., Larsen, P. L. and Riddle, D. L. (1998). Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. *Genetics* **150**, 129-155.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rothers, V. and Antebi, A. (2002). A putative hormonal signaling pathway influencing metabolism, reproductive development, and lifespan in *C. elegans*. *Dev. Cell* (in press).

Georgi, L. L., Albert, P. S. and Riddle, D. L. (1990). *daf-1*, a *C. elegans* gene controlling dauer larva development, encodes a novel receptor protein kinase. *Cell* **61**, 635-645.

Golden, J. W. and Riddle, D. L. (1984a). The *Caenorhabditis elegans* dauer larva: Developmental effects of pheromone, food and temperature. *Dev. Biol.* **102**, 368-378.

Golden, J. W. and Riddle, D. L. (1984b). A pheromone-induced developmental switch in *C. elegans*: temperature-sensitive mutants reveal a wild-type temperature-dependent process. *Proc. Natl. Acad. Sci. USA* **81**, 819-823.

Gonzalez, F. J., Ueno, T., Umeno, M., Song, B. J., Veech, R. L. and Gelboin, H. V. (1991). Microsomal ethanol oxidizing system: transcriptional and posttranscriptional regulation of cytochrome P450 CYP2E1. In *Advances in Biomedical Alcohol Research* (ed. H. Kalant, J. M. Khanna and Y. Israel). Pergamon Press.

Gotoh, O. (1998). Divergent structures of *Caenorhabditis elegans* cytochrome P450 genes suggest the frequent loss and gain of introns during the evolution of nematodes. *Mol. Biol. Evol.* **15**, 1447-1459.

Grenache, D. G., Caldicott, I., Albert, P. S., Riddle, D. L. and Politz, S. M. (1996). Environmental induction and genetic control of surface antigen switching in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **93**, 12388-12393.

Gunther, C. V., Georgi, L. L. and Riddle, D. L. (2000). A *Caenorhabditis elegans* type I TGF-β receptor can function in the absence of type II kinase to promote larval development. *Development* **127**, 3337-3347.

Hart, A. C., Sims, S. and Kaplan, J. M. (1995). Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* **378**, 82-85.

Hsin, H. and Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of *C. elegans*. *Nature* **399**, 362-366.

Inoue, T. and Thomas, J. H. (2000). Targets of TGF-β signaling in *C. elegans* dauer formation. *Dev. Biol.* **217**, 192-204.

Kazuyoshi, T., Kazuyoshi, U., Minoru, T., Chie, K. and Lea, R. W. (1999). Neurosteroid biosynthesis in vertebrate brains. *Comp. Biochem. Physiol. C: Comp. Pharmacol. Toxicol.* **124**, 121-129.

Kenyon, C., Chang, J., Gensch, E., Runder, A. and Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366**, 461-464.

Kimura, K. D., Tissenbaum, H. A., Liu, Y. and Ruvkun, G. (1997). *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* **277**, 942-946.

King, K. V. (1998). *Signaling Components in Development and Life Span Determination in* C. elegans. Ph. D. Thesis. Division of Biological Sciences, University of Missouri, Columbia.

Klass, M. R. and Hirsh, D. (1976). Non-aging developmental variant of *Caenorhabditis elegans*. *Nature* **260**, 523-525.

Lakowski, B. and Hekimi, S. (1996). Determination of life-span in *Caenorhabditis elegans* by four clock genes. *Science* **272**, 1010-1013.

Lakowski, B. and Hekimi, S. (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **95**, 13091-13096.

Larsen, P. L., Albert, P. S. and Riddle, D. L. (1995). Genes that regulate both development and longevity in *Caenorhabditis elegans*. *Genetics* **139**, 1567-1583.

Lin, K., Dorman, J. B., Rodan, A. and Kenyon, C. (1997). *daf-16*: an HNF-3/Forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* **278**, 1319-1322.

Matyash, V., Geier, C., Henske, A., Mukherjee, S., Hirsh, D., Thiele, C., Grant, B., Maxfield, F. R. and Kurzchalia, T. V. (2001). Distribution and transport of cholesterol in *Caenorhabditis elegans*. *Mol. Biol. Cell* **12**, 1725-1736.

Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocrine Rev.* **9**, 295-318.

Morris, J. Z., Tissenbaum, H. A. and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. *Nature* **382**, 536-539.

Nelson, D. (1998). Metazoan cytochrome P450 evolution. *Comp. Biochem. Physiol. C* **121**, 15-22.

Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D. et al. (1999). The Neiman-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J. Biol. Chem.* **274**, 9627-9635.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A. and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* **389**, 994-999.

Patterson, G. I., Koweek, A., Wong, A., Liu, Y. and Ruvkun, G. (1997). The DAF-3 Smad protein antagonizes TGF-β-related receptor signaling in the *Caenorhabditis elegans* dauer pathway. *Genes Dev.* **11**, 2679-2690.

Pierce, S. B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S. A., Buchman, A. R., Ferguson, K. C., Heller, J., Platt, D. M., Pasquinelli, A. A. et al. (2001). Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family. *Genes Dev.* **15**, 672-686.

Riddle, D. L. and Albert, P. S. (1997). Genetic and Environmental Regulation of Dauer Larva Development. In *C. elegans II* (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 739-768. NY: Cold Spring Harbor Laboratory Press.

Ren, P.-f., Lim, C.-S., Johnsen, R., Albert, P. S., Pilgrim, D. and Riddle, D. L. (1996). Control of *C. elegans* larval development by neuronal expression of a TGF-β homolog. *Science* **274**, 1389-1391.

Rushforth, A. M. and Anderson, P. (1996). Splicing removes the *Caenorhabditis elegans* transposon Tc1 from most mutant pre-messenger RNAs. *Mol. Cell. Biol.* **16**, 422-429.

Schackwitz, W. S., Inoue, T. and Thomas, J. H. (1996). Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans*. *Neuron* **17**, 719-728.

Sluder, A. E., Mathews, S. W., Hough, D., Yin, V. P. and Maina, C. V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. *Genome Research* **9**, 103-120.

Sym, M., Basson, M. and Johnson, C. (2000). A model for Niemann-Pick type C disease in the nematode *Caenorhabditis elegans*. *Curr. Biol.* **10**, 527-530.

Tremblay, Y., Fleury, A., Beaudoin, C., Vallee, M. and Belanger, A. (1994). Molecular cloning and expression of Guinea pig cytochrome P450 P450c17 cDNA (steroid 17α-hydroxylase/17,20 lyase): tissue distribution, regulation, and substrate specificity of the expressed enzyme. *DNA and Cell Biology* **13**, 1199-1212.

Whitefield, G. K., Jurutka, P. W., Haussler, C. A. and Haussler, M. R. (1999). Steroid hormone receptors: evolution, ligands, and molecular basis of biological function. *J. Cell. Biochem.* **Suppl. 32/33**, 110-122.

White, J. G., Southgate, E., Thomson, J. N. and Brenner, F. R. S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Phil. Trans. R. Soc. Lond. B* **314**, 1-340.

Williams, B. D., Schrank, B., Huynh, C., Shownkeen, R. and Waterson, R. H. (1992). A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* **131**, 609-624.

Wolkow, C. A., Kimura, K. D., Lee, M.-S. and Ruvkun, G. (2000). Regulation of *C. elegans* life-span by insulin like signaling in the nervous system. *Science* **290**, 147-150.