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A Hormonal Signaling Pathway Influencing *C. elegans* Metabolism, Reproductive Development, and Life Span

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Summary

During *C. elegans* development, animals must choose between reproductive growth or dauer diapause in response to sensory cues. Insulin/IGF-I and TGF-β signaling converge on the orphan nuclear receptor *daf-12* to mediate this choice. Here we show that *daf-9* acts downstream of these inputs but upstream of *daf-12*. *daf-9* and *daf-12* mutants have similar larval defects and modulate insulin/IGF-I and gonadal signals that regulate adult life span. *daf-9* encodes a cytochrome P450 related to vertebrate steroidogenic hydroxylases, suggesting that it could metabolize a DAF-12 ligand. Sterols may be the *daf-9* substrate and *daf-12* ligand because cholesterol deprivation phenocopies mutant defects. Sensory neurons, hypodermis, and somatic gonadal cells expressing *daf-9* identify potential endocrine tissues. Evidently, lipophilic hormones influence nematode metabolism, diapause, and life span.

Introduction

Metazoans have evolved intricate and dynamic endocrine networks that coordinate their energy needs for development and reproduction (Schwartz et al., 2000). Reproductive development in *C. elegans* is dramatically regulated by the environment. In favorable conditions, worms develop successively from embryo through four larval stages (L1–L4) to reproductive adult in 3 days, and then live for another 2–3 weeks. In unfavorable conditions, they instead arrest as sexually immature larvae and enter an alternate third stage, the dauer diapause (Riddle and Albert, 1997). This choice entails organism-wide coordination suggestive of endocrine regulation. In particular, sensory cues of dwindling food, warm temperatures, and high concentrations of crowding pheromone shift metabolism toward fat and carbohydrate storage, induce stress-protective enzymes, modify behavior, and promote dauer morphogenesis (Riddle and Albert, 1997). These adaptations enable dauer larvae to survive harsh conditions. Remarkably, they often live months without food, and upon return to favorable environments recover to fertile adults, implying an underlying mechanism to prolong life span. As in many other species, the life span of nematodes that have already bypassed diapause is also extended by caloric restriction (Klass, 1977).

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Products of the dauer formation genes (Daf) evaluate sensory information and subsequently select programs of diapause or reproductive growth. Dauer-defective (Daf-d) mutants bypass diapause, while dauer-constitutive (Daf-c) mutants inappropriately choose diapause, irrespective of sensory cues (Riddle and Albert, 1997). Molecular, genetic, and cellular analyses of the Daf loci reveal a network of conserved neuroendocrine pathways, including serotonergic (Sze et al., 2000), insulin/IGF-I (Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997; Ogg and Ruvkun, 1998; Paradis et al., 1999; Paradis and Ruvkun, 1998; Pierce et al., 2001), TGF-β (Estevez et al., 1993; Georgi et al., 1990; Inoue and Thomas, 2000; Ogg et al., 1997; Patterson et al., 1997; Ren et al., 1996; Schackwitz et al., 1996), and cGMP signaling (Birnby et al., 2000; Coburn et al., 1998) that form logical circuits coupling sensory inputs to behavioral, metabolic, and developmental outputs. These pathways converge on orphan nuclear receptor DAF-12 in cellular targets (Antebi et al., 2000). *daf-12* mutants display either Daf-d or Daf-c phenotypes as well as a heterochronic delay in the expression of L3 programs (Antebi et al., 1998; Riddle et al., 1981), revealing an instructive role in the choice of L3 fates. *daf-12*'s molecular identity suggests that lipophilic hormones could ultimately instruct this decision (Antebi et al., 2000). However, components of hormone metabolism have not yet been found, though homologs exist in the genome (*C. elegans* Sequencing Consortium, 1998).

Insulin/IGF-I signaling also regulates other nematode life history traits, including fertility, reproductive schedule, and, most notably, adult life span (Friedman and Johnson, 1988; Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995; Tissenbaum and Ruvkun, 1998). For example, reduced activity of the DAF-2 insulin/IGF-I receptor extends life span 2-fold, an increase dependent on the DAF-16 FORKHEAD/FKHR/AFX transcription factor (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Longevity measurably correlates with oxidative protection from catalase and superoxide dismutase (Honda and Honda, 1999; Larsen, 1993; Taub et al., 1999). *daf-12* modulates this longevity, weakly suppressing some long-lived mutants (*daf-2* class 1), but enhancing others (*daf-2* class 2). Alone, *daf-12* modestly shortens life span (Gems et al., 1998; Larsen et al., 1995). The gonad also regulates nematode longevity and *daf-12* functions in this pathway as well (Hsin and Kenyon, 1999). These interactions imply potential lipophilic hormonal influences on life span.

daf-9 was previously identified as a mutant with Daf-c phenotypes (Albert and Riddle, 1988) and delayed heterochrony in the somatic gonad (Antebi et al., 1998). Here we report that *daf-9* and *daf-12* share similar phenotypes and are closely coupled in pathways controlling metabolism, reproductive development, and life span. *daf-9* encodes a cytochrome P450, suggesting hormonal regulation through *daf-12*. These findings reveal an important link within *C. elegans* endocrine networks.

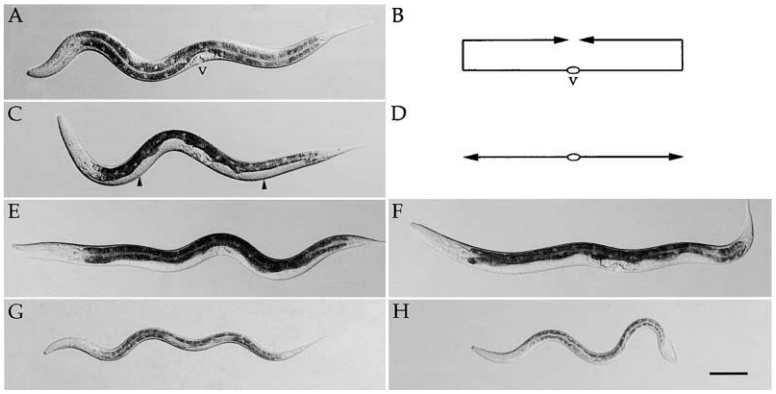


Figure 1. Comparative Mig and Daf-c Phenotypes

(A) Wild-type L4 larva. The gonad, obscured here by the intestine, packs into the body cavity as two U-shaped arms, as drawn schematically in (B). v, vulva. (C) Wild-type grown on cholesterol-deficient medium. The gonadal Mig phenotype is seen as a ventral patch of white tissue (arrowheads) that extends into head and tail, as drawn schematically in (D). (E) *daf-9(rh50)* and (F) *daf-12(rh284)* Mig larvae. (G) *daf-9(dh6)* and (H) *daf-12(rh273)* L3 partial dauer larvae. Scale bar, 100 μM, left lateral aspect.

Results

daf-9 Regulates Reproductive Growth and Dauer Formation

To identify potential hormone metabolic genes, we searched for loci with phenotypes resembling *daf-12* mutants. *mig-8(rh50)* was previously isolated in screens for gonadal leader cell migration defective (Mig) mutants (Antebi et al., 1998). In *rh50* hermaphrodites, the distal tip cells, which pioneer gonadal outgrowth, fail to turn dorsally during L3 and erroneously migrate with unchanged direction (Figure 1E). This phenotype is interpreted as a heterochronic delay or arrest of stage-appropriate migration programs (Antebi et al., 1998). Later gonadal defects include delayed gonadoblast divisions, loose germ cells, miscued oocytes (data not shown), and small broods (Table 1). Occasionally mutants also fail to execute L3 intestinal programs on schedule and instead repeat L2 programs (data not shown). Notably, *rh50* is nearly indistinguishable from *daf-12(rh284)* (Figure 1F; Table 1), which affects a predicted ligand contact residue in the DAF-12 ligand binding domain (LBD) (Antebi et al., 2000).

From other genetic screens, two additional Mig alleles (class 1: *dh4*, *k182*) were isolated as well as several novel alleles (class 2: *dh6*, *dh7*, *dh8*, *dh9*, *dh10*, *dh11*) that confer a penetrant Daf-c phenotype. A previously identified Daf-c mutant, *daf-9(e1406)*, mapped nearby (Albert and Riddle, 1988) and failed to complement *mig-8*. We refer to it hereafter as *daf-9*.

Class 2 mutants develop through early stages but then arrest as L3 dauer larvae with complete penetrance (Table 1). Daf-c animals are partial rather than normal dauer larvae and resemble those formed in *daf-12(rh273)* (Figures 1G and 1H; Table 1), which also affects a predicted ligand contact residue. Like normal dauer larvae, partial dauers arrest somatic development and synthesize dauer alae, a lateral ridged cuticular tread. Unlike normal dauers, radial constriction of the body is incomplete, the pharynx pumps, and germ cells proliferate. One-third eventually resume development and molt to L4 and adult animals, most (29/33) of which belatedly synthesize dauer cuticle. A few animals retain the old cuticle as an outer sheath, much like dauers. Adults are infertile, with delayed or arrested gonadogenesis and gametogenesis. Other reproductive tissues, such as the vulva, are also developmentally arrested (data not shown).

To measure the effect of gene dose, class 1 allele *rh50* was put over deletion *mcDf2* and null allele *dh6*. No phenotypic enhancement was seen, i.e., no partial dauers formed in transheterozygotes at 20°C (Table 1). However, homozygous *rh50* cultures grown at 25°C contained 7% partial dauer larvae. Thus, *rh50* is influenced by temperature and may partly or selectively reduce gene function.

Cholesterol Deprivation Phenocopies *daf-9*

C. elegans requires cholesterol for normal development. We noticed that wild-type cultured in cholesterol-deficient media often displayed gonadal Mig phenotypes similar to *rh50* and *daf-12(rh284)* hypomorphs (Figure 1C; Table 1). A few animals also formed dauer-like larvae. We also cultured null alleles of three Daf-d loci, *daf-3(mgDf90)*, *daf-16(mgDf50)*, and *daf-12(rh61rh411)* on cholesterol-deficient media. DAF-3/SMAD and DAF-16/FORKHEAD/FKHR/AFX mediate transcriptional outputs of TGF-β and insulin/IGF-I signaling, respectively (Lin et al., 1997; Ogg et al., 1997; Patterson et al., 1997). Mig and Daf-c phenotypes were observed in *daf-3* and *daf-16* but not in *daf-12* mutants (Table 1). Cholesterol addition rescued these phenotypes (data not shown). These experiments localize the cholesterol-sensitive step downstream of *daf-3* and *daf-16*, but upstream of *daf-12* in the dauer pathway, the same position as *daf-9* in genetic epistasis experiments (below). Moreover, cholesterol deprivation enhanced the Daf-c phenotypes of *rh50* and *rh284* at 20°C, which normally appear with low penetrance at 25°C (Table 1). These observations suggest that cholesterol availability influences signaling in the dauer pathway proximal to *daf-9* and *daf-12*.

Genetic Epistasis

To order *daf-9* within the dauer pathway, we performed genetic epistasis experiments with Daf-c;Daf-d double mutants. Class 2 alleles, *dh6* and *dh10*, were used to construct double mutants with *daf-3*, *daf-16*, and *daf-12* Daf-d null alleles. In reproductive growth conditions, *daf-9* alone forms partial dauer larvae. *daf-12* efficiently suppresses this phenotype while *daf-3* and *daf-16* do not (Table 1), placing *daf-9* downstream or parallel to *daf-3* and *daf-16*, but upstream of *daf-12*. In dauer inducing conditions, *daf-9* mutants alone form normal dauer

Table 1. Phenotypes of *daf-9*, *daf-12*, and Double Mutants

Genotype, Growth Condition[a]	Distal Tip Cell Reflection[b] (%)	Daf-c Partial Dauer Formation[c] (%)	Normal Dauer Formation[d] (%)	Brood Size[e]
N2	100	0	>5	334 ± 47
N2, minus chol[f]	74	6	—	—
daf-9(*dh4*)	74	0	>5	65 ± 42
dh4, 25°C	84	0	—	—
rh50	11	0	>5	86 ± 19
rh50, 25°C	11	7	—	—
rh50/*mcDf2*	7	0	—	—
rh50/*dh6*	25	0	—	—
rh50, NG minus chol[g]	1	53	—	—
k182	95	0.4	>5	201 ± 52
k182, 25°C	98	1	—	—
daf-12(*rh284*)	49	0	>5	67 ± 27
rh284, 25°C	57	10	—	—
rh284, NG minus chol[g]	0	73	—	—
daf-12(*rh61rh411*)	100	0	0	—
rh61rh411, minus chol[f]	100	0	—	—
daf-3(*mgDf90*)	100	0	0	—
mgDf90, minus chol[f]	55	3.1	—	—
daf-16(*mgDf50*)	100	0	0	—
mgDf50, minus chol[f]	70	4	—	—
daf-9(*dh6*)	0	22[h], 100[i]	>5	0
dh6 rh61rh411	100	0	0	—
dh6;*mgDf50*	0	21 (100)[h]	0	0
daf-9(*dh10*);*mgDf90*	0	25 (100)[h]	>5	0
daf-12(*rh273*)	24	18	>5	—
rh273;*mgDf50*	—	7	0	—
rh273;*mgDf90*	—	99	>5	—

[a] Grown at 20°C on NGM unless indicated otherwise.
[b] Hermaphrodite distal tip cells that turn in L3, n > 50 cells.
[c] Partial dauers formed under reproductive growth conditions, i.e., Daf-c (Antebi et al., 1998). n > 100 animals, except *rh50*/*mcDf2* (n = 57), *rh50*/*dh6* (n = 55), and *k182*, 25°C (n = 96).
[d] Percent that form normal dauer larvae (estimated) from crowded cultures grown to exhaustion on NGM (dauer-inducing conditions). n > 600 animals.
[e] Mean brood size per hermaphrodite (±SEM). n > 7 broods.
[f] Grown on cholesterol deficient media (Yochem et al., 1999).
[g] Grown on NGM without added cholesterol.
[h] Percent Daf-c F1 progeny from mothers heterozygous for *daf-9*. In parenthesis, estimated Daf-c frequency of *daf9*;*daf-3* or *daf-9*;*daf-16* homozygotes.
[i] Measured from *daf-9* homozygotes that have lost the *daf-9*(+); *sur-5::gfp* extrachromosomal array *dhEx38*.

larvae. Again, *daf-12* completely suppresses this phenotype, while *daf-3* does not. However, *daf-9;daf-16* cultures produce partial rather than normal dauer larvae, showing that *daf-16* also acts independently of *daf-9.* In epistasis experiments, the *daf-12(rh273*) LBD mutant behaves like *daf-9* nulls, downstream or parallel to *daf-3* and *daf-16* (Table 1). However, *daf-3* enhances the penetrance of the *rh273* Daf-c phenotype from 18% to 99%, suggesting some interaction.

daf-9 Regulates Fat Storage

In dauer-inducing conditions, wild-type shifts metabolism from energy utilization to storage. L2 predauers (L2d) darken as granules in intestine and epidermis store fat and complex carbohydrates (Riddle and Albert, 1997). Sudan black, which stains fat, does not appreciably stain wild-type larvae grown under reproductive growth conditions. However, it darkly stains dauers and Daf-c mutants from both insulin/IGF-I (e.g., *daf-2*) and TGF-β (e.g., *daf-7*) pathways (Kimura et al., 1997), a phenotype blocked by Daf-d mutants, *daf-16* and *daf-3*, respectively (Ogg et al., 1997).

In reproductive growth conditions, *daf-9* L2d larvae also stain darkly with Sudan black (Figure 2C), suggesting they initialize the shift toward fat storage. By contrast, after they complete dauer morphogenesis, resume feeding, and attempt recovery, they become somewhat lighter (by DIC microscopy), suggesting that some energy stores are mobilized during subsequent growth. Fat storage is independent of *daf-3* and *daf-16*, but requires *daf-12*. *daf-9* L2d animals stain as darkly as double mutants with *daf-3* and *daf-16*, whereas *daf-12 daf-9* double mutants are as light as *daf-12* null mutants alone (Figures 2E, 2F, and 2H). Like *daf-9*, *daf-12(rh273)* Daf-c LBD mutants themselves display the dark staining phenotype (Figure 2G). Finally, *daf-12* null mutants do not suppress the dark staining phenotypes of *daf-7* (Figure 2H) and *daf-2* (data not shown). Therefore, outputs of TGF-β and insulin/IGF-I signaling can regulate fat storage independently of *daf-12*.

daf-9 Encodes a Cytochrome P450

daf-9 was previously mapped to the X chromosome between *mec-2* and *mup-2* (Antebi et al., 1998) and further localized using established left endpoints of *stDf1* and *mcDf2* deletions (M. Labouesse, personal

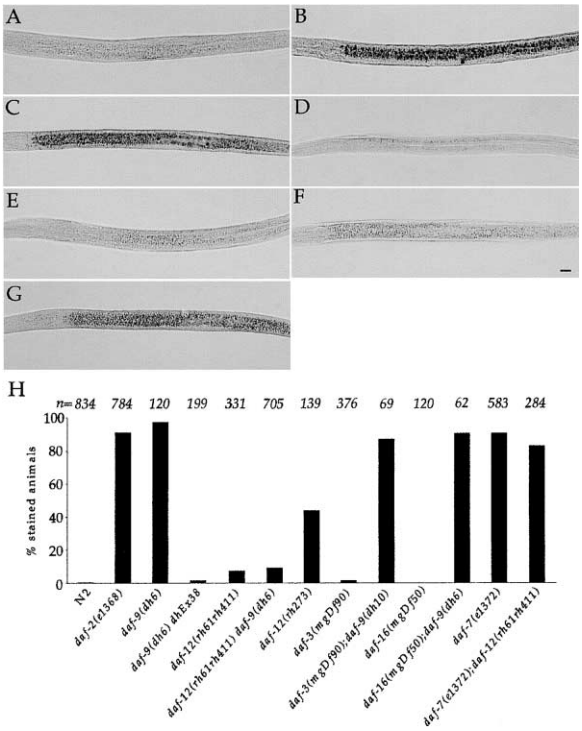


Figure 2. Sudan Black Staining of L2/L3 Larvae under Reproductive Growth Conditions

(A) Wild-type (B) *daf-2(e1368)* (C) *daf-9(dh6)* (D) *dh6* with *daf-9(+)* extrachromosomal array *dhEx38* (E) *dh6 daf-12(rh61rh411)* (F) *daf-12(rh61rh411)* (G) *daf-12(rh273).* Scale bar, 10 μM. (H) Quantitative analysis of Sudan black staining (n = number of animals). Staining was carried out as described (Ogg and Ruvkun, 1998). *e1368* and *e1372* were grown at 25°C.

communication). An activity rescuing the *rh50* Mig phenotype was narrowed to cosmid T13C5 (Figure 3A) and the sequence of candidate genes from *daf-9* mutants localized mutations to gene T13C5.1. The deduced coding region comprises 9 exons spanning 2.5 kb of genomic DNA (Figure 3B). Two SL1-spliced transcripts of 1.8 and 1.4 kb encode proteins of 536 (DAF-9B) and 376 (DAF-9C) amino acids, respectively. A longer isoform (DAF-9A) is not SL1 spliced and contains an additional N-terminal exon (data not shown; Jia et al., 2001).

DAF-9 is homologous to cytochrome P450 (CYP) enzymes, a large superfamily of monooxygenases that use the cofactor heme (Hasemann et al., 1995). CYPs metabolize steroids, bile acids, fatty acids, arachidonates, and xenobiotics. Eukaryotic CYPs are mitochondrial or microsomal. DAF-9 more closely resembles the latter. Alignment with CYPs of known structure (Hasemann et al., 1995; Williams et al., 2000) shows that DAF-9 contains core motifs required for heme binding and coordination, folding and catalysis, as well as variable regions involved in substrate recognition (Figure S1, Supplemental Data [http://www.developmentalcell.com/cgi/content/full/1/6/841/DC1]).

Blast analysis reveals that DAF-9 (a.k.a. CYP22A1) is most similar to the CYP2 family, whose member activities include hormone metabolism (rabbit 2C5 21-progesterone hydroxylase [Pendurthi et al., 1990]), fatty acid metabolism (rabbit 2C2 laurate omega-1 hydroxylase [Leighton et al., 1984]), or xenobiotic detoxification (human 2C19 mephenytoin 4-hydroxylase [Romkes et al., 1991]). In fact, DAF-9 is more similar to these vertebrate proteins than to its closest nematode relatives (e.g., CYP23A1). Phylogenetic analysis on nearly all eighty *C. elegans* CYPs also suggests that DAF-9 may be more related to vertebrate CYPs than to *C. elegans* proteins (Figure 3C) (Gotoh, 1998; Nelson, 1998). Less similar are mammalian steroidogenic hydroxylases, CYP17 and CYP21 (Greenspan and Baxter, 1994) and the *Drosophila* CYP18 (Bassett et al., 1997). When vertebrate CYPs are queried by BLAST against Wormpep, which contains the known and predicted *C. elegans* ORFs, other *C. elegans* CYPs (e.g., CYP34A4) are often more similar than DAF-9. These relationships may arise because DAF-9 and other *C. elegans* CYPs are closely rooted in phylogenetic trees.

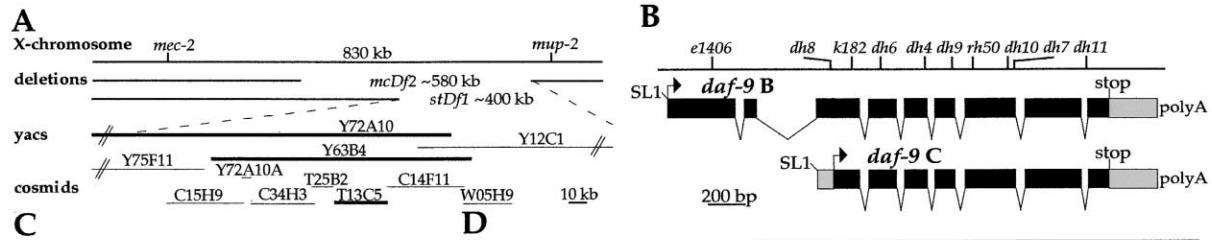
Molecular Lesions

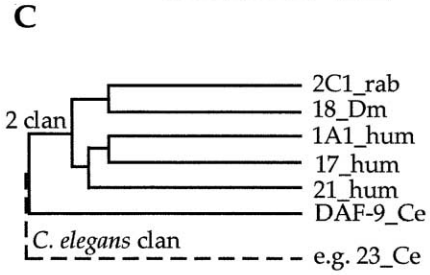
Class 2 alleles are the stronger loss of function and consist of premature stop codons (*dh6*, *dh7*, *dh9*), a splice acceptor mutation (*dh11*), a frameshift mutation (*e1406*), or missense mutations within conserved residues (*dh8, dh10*) (Figures 3D and S1, Supplemental Data [http://www.developmentalcell.com/cgi/content/full/1/6/841/DC1]). In particular, *dh6* (Q225stop) is a candidate molecular null that disrupts all isoforms, whereas reference allele *e1406* disrupts isoforms A and B only. Missense allele *dh8* (G156E) affects a conserved glycine in the B′C loop, which comprises part of the active site and is implicated in substrate binding (Hasemann et al., 1995). *dh10* (E394K) reverses the charge of an invariant glutamate that forms an important salt bridge required for heme binding, folding, and enzymatic activity (Yoshikawa et al., 1992). Class 1 alleles (*rh50*, *dh4*, *k182*) are missense lesions. Interestingly, *dh4* (L288F) affects helix G, which influences substrate recognition (Hasemann et al., 1995). Similarly, *rh50* (D334N) affects a conserved aspartate in the I helix; an analogous mutation in aromatase (E302N) perturbs substrate binding (Graham-Lorence et al., 1991).

Expression Pattern

To analyze *daf-9* expression, we constructed a functional *daf-9::gfp* gene fusion that rescues mutant phenotypes and examined extrachromosomal (*dhEx61*, *dhEx66*) and integrated lines (*dhIs59*, *dhIs64*). *daf-9* was strikingly expressed in a ventral pair of bilateral neurons identified as the IL1Vs or URAVs in the anterior ganglia (Figure 4A). DAF-9 was perinuclear, consistent with residence in endoplasmic reticulum. Neuronal expression first appeared shortly before hatch, increased greatly during larval development, and perdured in reproductive and postreproductive adults.

By mid-L2, DAF-9 also appeared in the cytoplasm of the hypodermis (Figure 4B), the syncitial epidermis surrounding the worm, but was absent from midline epidermal seam cells. Levels peaked around the L2 molt and diminished during L4. In some cases, transient expression was seen in L3 vulval blast cells. *daf-9* was also expressed within the hermaphrodite spermatheca (Figure 4C) starting in late L4 larvae, and continuing even in old adults. In some extrachromosomal lines, sporadic





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Allele	Change	Location in Protein	Predicted Consequence
Class 1			
rh50	D334N	I	substrate binding
dh4	L288F	G	substrate binding
k182	S206K	β3-1	unknown
Class 2			
e1406	L97frame shift	A	truncation
dh6	Q225stop	E	truncation, null
dh7	W404stop	β6-1	truncation
dh8	G156E	B C loop	heme, substrate binding
dh9	Q316stop	β5-1	truncation
dh10	E394K	K	folding, heme binding, catalysis
dh11	splice acceptor	β3–3	truncation

Figure 3. The *daf-9* Locus, Structural and Phylogenetic Relationships

(A) Genetic map (above) and physical map (below) showing rescuing YACs and cosmids (bold).

(B) Structure of the *daf-9* gene. Bar, genomic DNA with the position of mutations. Two SL1-spliced mRNA transcripts, *daf-9B* and *C*, are indicated. Black arrow, initiator methionine; black box, exons; gray box, 5′ and 3′ UTRs; polyA, polyadenylation site.

(C) Phylogenetic tree showing the relation of DAF-9 to its closest nematode and vertebrate relatives (Nelson, 1998).

(D) *daf-9* mutations.

weak expression was seen in the excretory cell, a few unidentified head neurons, and vulval muscles. In males, *daf-9* was expressed in IL1V/URAVs and hypodermis but not somatic gonad. In dauer larvae, *daf-9* was strongly expressed in IL1V/URAV and specifically extended into axonal but not dendritic processes. No hypodermal expression was evident in dauers. In post-dauer stages, *daf-9* was expressed in a pattern similar to reproductively growing animals, except expression was absent in the hypodermis.

The dramatic expression of *daf-9* within IL1V/URAVs suggested a possible role for these neurons in promoting reproductive growth. To test this hypothesis, they were ablated in newly hatched L1 larvae by laser microsurgery in *dh6* containing *dhIs64*, a *daf-9::gfp* chromosomal integrant. All animals reached reproductive maturity (n = 41) and often showed compensatory *daf-9* upregulation in the hypodermis. A few formed dauer-like larvae (4/41) that promptly recovered. We conclude that *daf-9* expression in other tissues suffices to bypass diapause.

Life Span Phenotypes

We measured the adult life span of different *daf-9* alleles in single- and double-mutant combinations with *daf-2*, *daf-12*, and *daf-16* at different temperatures (Figure 5; Table 2). Class 1 allele *daf-9(rh50)* alone had a mean life span slightly shorter than wild-type. In addition, *rh50* slightly reduced the mean life span of *daf-2(e1368)* but increased the last quartile mean and maximum life span of *daf-2(e1370)* at 22.5°C (Figure 5A; Table 2). At 15°C, however, *rh50* actually diminished *daf-2* longevity. *daf-12* null mutants interact with *daf-2* class 1 and 2 mutants in a similar manner (Gems et al., 1998).

With *daf-9* class 2 alleles (*dh6*, *dh8*, *dh10*, *e1406*), we examined partial dauer larvae that had recovered to adult. These adults appeared dauer-like with developmental defects in reproductive tissues. Although overall mean life spans of most of these mutants were slightly lower than wild-type, maximum life spans were generally increased at 15°C. For example, *e1406* resulted in a 52% increase in maximum life span. Only this allele showed a significant 24% increase for the last quartile mean life span, though similar trends were seen with other alleles (Figure 5B; Table 2). Life span extension was also observed in *e1406* doubles with *daf-16*, suggesting that longevity due to *e1406* is *daf-16* independent. By contrast, *daf-12(rh61rh411)* largely suppressed *e1406* longevity (Figure 5C; Table 2). Therefore, *daf-9* longevity appears predominantly *daf-12(+)* dependent. Similar trends were seen with *dh8* double mutants (data not shown).

Besides genetic influences, specific tissues of the nematode affect life span. In particular, ablation of the two germline precursors, Z2 and Z3, by laser microsurgery extends wild-type mean life span by 64% (Hsin and Kenyon, 1999). Additional removal of the somatic gonadal precursors, Z1 and Z4, abrogates this extension, suggesting that opposing signals from germline and somatic gonad could regulate longevity. Moreover, longevity is *daf-12(+)* dependent (Hsin and Kenyon, 1999). Germline ablations in *daf-12* mutants do not extend life span, but somatic gonad ablations further shorten life span, suggesting that somatic gonadal but not germline signaling is intact in mutants.

We asked whether germline ablations in *daf-9* would behave similarly. Neither class 1 *rh50* nor class 2 *dh6* germline ablated animals lived longer than untreated controls (Figures 5E and 5F). By contrast, germline ablations in *dh6* containing a *daf-9(+)* rescuing array

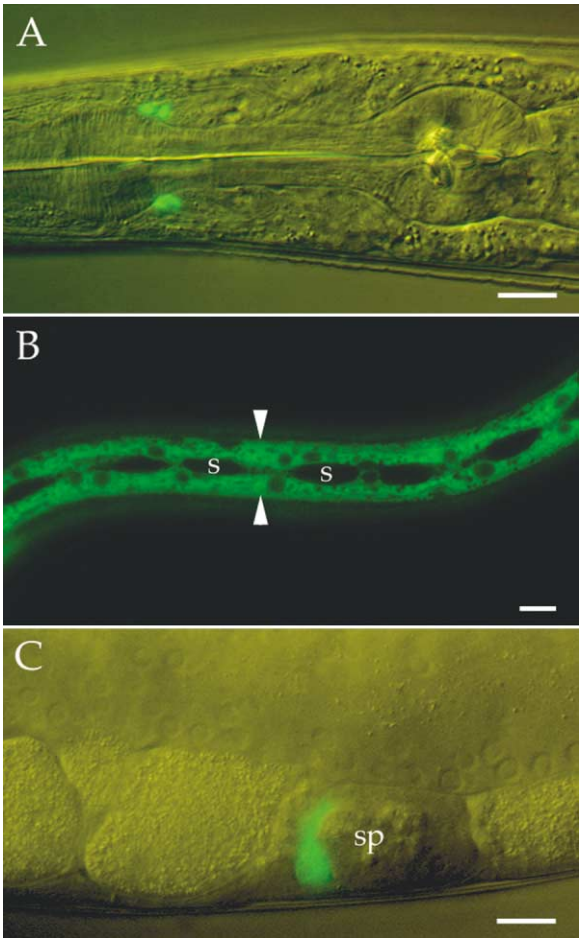

Figure 4. *daf-9::gfp* Expression Pattern

(A) Adult IL1V/URAV neurons (B) L3 syncitial hypodermis, hyp7 (arrowheads), seam cells (s).

(C) Adult spermatheca, sperm (sp).

Scale bar, 10 μM. (A) ventral aspect; (B and C) left lateral aspect.

(*dhEx24*) as well as N2 lived 22% and 47% longer, respectively (Table 2; Figure 5D). We conclude that longevity of germline-ablated animals depends on *daf-9(+)*. Nor did ablation of the somatic gonad shorten life span any more than germline ablations for either *rh50* or *dh6*. Thus, longevity signals from the intact somatic gonad might also depend on *daf-9(+)*. Similar results were obtained at 22.5°C and with allele *dh4* (data not shown).

Discussion

The Hormone Hypothesis

The discovery that *daf-9* encodes a cytochrome P450 and *daf-12* a nuclear receptor together provides substantial evidence for hormonal signaling by lipophilic molecules in *C. elegans*. Phylogenetic analysis by methods of distance and parsimony suggests that DAF-9 is most related to the CYP2 family (Gotoh, 1998; Nelson, 1998). Family members typically metabolize steroid hormones, fatty acids, as well as xenobiotics. Although the DAF-9 substrate(s) is still unknown, several pieces of evidence suggest a close link to sterol metabolism. First, wild-type grown on cholesterol-deficient media phenocopy *daf-9* Mig and Daf-c defects. By genetic epistasis tests, the cholesterol-sensitive step lies at the same point as *daf-9* in the dauer pathway. Reduced cholesterol also enhances Daf-c phenotypes of *daf-9* and *daf-12* hypomorphs. Finally, DAF-12 clusters in a branch of the nuclear receptor superfamily regulated by sterols (e.g., CAR-β, androstanols; PXR, pregnanes; VDR, vitamin D) (Antebi et al., 2000). By inference, DAF-9 may be a primordial sterol-metabolic enzyme and DAF-12 its sterol-regulated nuclear receptor partner. Several sterol metabolites are found in *C. elegans* but none have an ascribed function (Chitwood, 1999). Ultimately, identification of hormone and biosynthetic intermediates await biochemical analysis.

daf-9 Forms a Phenotypic Map onto the *daf-12* LBD

daf-9 mutants specifically resemble certain *daf-12* LBD mutants that affect predicted ligand contact sites (Antebi et al., 2000), consistent with a biochemical function related to ligand/receptor interactions. *daf-9* mutants fall into two phenotypic classes. Class 1 mutants exhibit delayed heterochronic phenotypes, notably delayed gonadal leader cell migrations, and no or low penetrance Daf-c phenotypes. Class 2 mutants arrest as partial dauer larvae that slowly recover to sterile adults.

Molecular analysis shows that class 2 mutations are null or severe loss-of-function alleles, whereas class 1 alleles are missense lesions, the majority of which cluster in putative substrate recognition motifs. Presumably, class 1 lesions reduce affinity for DAF-9 substrate(s). We suggest that gonadal Mig and other heterochronic phenotypes arise from an inhibition of third-stage reproductive programs. There may be an additional failure to actively promote reproductive programs. Low levels of DAF-9 activity could suffice to bypass dauer formation while high levels may be required to bypass arrest of reproductive programs. Alternately, DAF-9 could have selectively mutable activities for reproductive and dauer development. As precedents, different levels of CYP21 activity result in variant syndromes of adrenal hyperplasia, while CYP17 participates in both cortisol and estrogen biosynthesis (Greenspan and Baxter, 1994).

daf-9 Couples TGF-β and Insulin/IGF-I Signals to *daf-12*

Our genetic experiments reveal that *daf-12* effectively suppresses most *daf-9*-visible phenotypes and enables animals to reach reproductive maturity, showing that *daf-9* acts through *daf-12(+)*. TGF-β and insulin-like branches show somewhat more complex epistasis relations, which imply they converge on lipophilic hormone signaling for some functions but diverge for others. For example, in reproductive growth conditions, the partial dauer Daf-c phenotypes of *daf-9* and *daf-12* LBD mutants are epistatic to *daf-3*/SMAD and *daf-16*/FORKHEAD/AFX/FKHR Daf-d mutants, showing convergence for many aspects of dauer formation. However, under dauer-inducing conditions *daf-16* is required in all genotypes for complete dauer morphogenesis, specifically for pharyngeal remodeling and total radial constriction (Ogg et al., 1997). Moreover, *daf-16(+)* is the major output for life

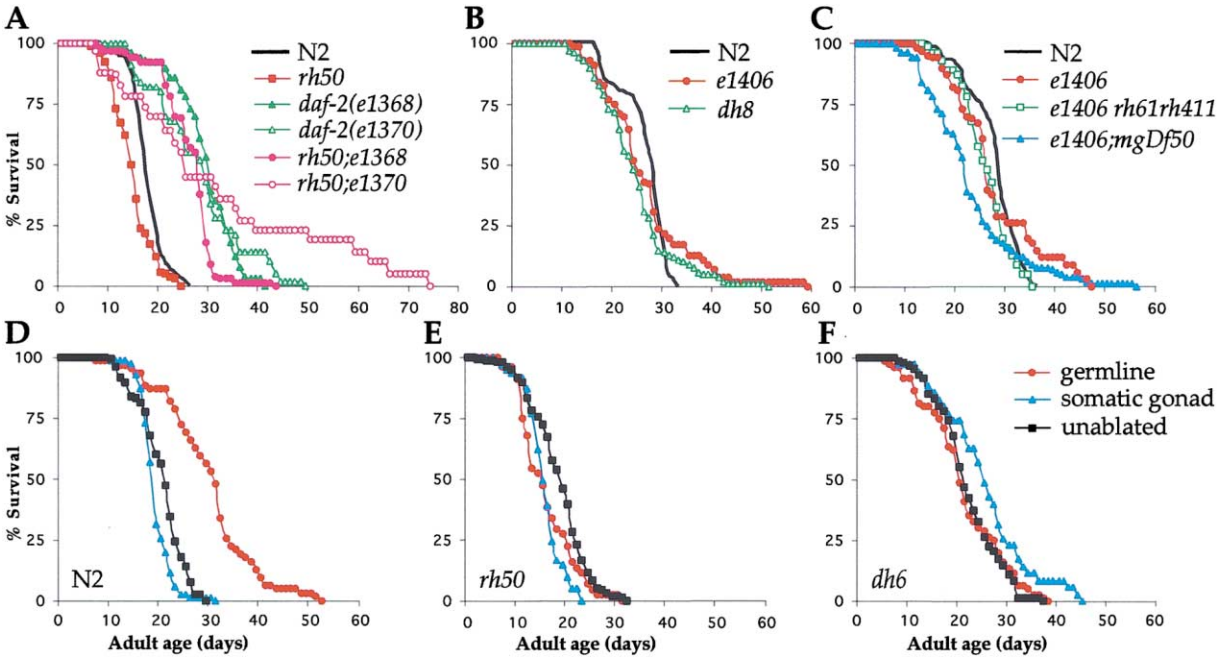

Figure 5. Life Span Assays

(A) Interactions of *rh50* with *daf-2* at 22.5°C. (B) Class 2 *daf-9* alleles *e1406* and *dh8* at 15°C, (C) *e1406* doubles with *daf-12(rh61rh411)* and *daf-16(mgDf50)* at 15°C. (D) Life spans of Z2, Z3 (germline) ablated and Z1, Z4 (somatic gonad) ablated wild-type, (E) *rh50*, and (F) *dh6* animals at 20°C. Z1, Z4 ablations result in the death of all four gonadal precursors.

span regulation by *daf-2* (Kenyon et al., 1993). *daf-3(+)* mediates pre-dauer foraging behavior, as seen in *daf-7* mutants (Thomas et al., 1993). Finally, fat storage is evidently regulated independently by insulin-like, TGF-β, and lipophilic hormone signaling pathways (this work). At the molecular level, DAF-12, DAF-16, and DAF-3 may act together at some promoters and independently at others.

The DAF-2 insulin/IGF-I receptor and the DAF-4 type II TGF-β receptor work cell nonautonomously to control metabolism and diapause, revealing a secondary signal downstream of these pathways (Apfeld and Kenyon, 1998; Inoue and Thomas, 2000; Wolkow et al., 2000). It seems plausible that DAF-9 defines the secondary signal for diapause. A simple model is that *daf-9* integrates inputs from TGF-β and insulin/IGF-I pathways and produces or degrades a hormone that regulates DAF-12 transcriptional complexes. It is also possible that another undiscovered layer of signaling lies between these pathways and *daf-9*. Conceivably, vertebrate insulin/IGF-I action might also have analogous hormonal outputs, and some forms of type II diabetes may reflect the dysregulation of vertebrate DAF-9 or DAF-12 analogs.

daf-9 Expression Suggests Endocrine Regulation

Whereas *daf-9* is expressed in only a few cells and tissues, namely IL1V/URAV neurons, hypodermis, and spermatheca, *daf-12* is expressed within virtually all cells during larval development (Antebi et al., 2000). By inference, *daf-9*-expressing cells represent endocrine tissues that regulate organismal commitments through DAF-12. *daf-9* and *daf-12* are also expressed in congruent tissues, which suggests potential intracrine regulation as well.

Although neuronal *daf-9* may be important for normal diapause regulation, it is not essential, since ablation of the IL1V/URAV neurons rarely causes dauer arrest. Compensatory upregulation in the hypodermis results, showing intercellular communication between these tissues. Other observations point to a role for hypodermis. Hypodermal expression ensues by mid-L2, the proposed point of third stage commitments to reproductive growth (Antebi et al., 1998). Conversely, *daf-9* is downregulated in the hypodermis during entry into diapause. Perhaps the hypodermis converts peripheral hormone to an active form. Mosaic analysis should resolve the relative contributions of different *daf-9*-expressing tissues.

daf-9/*daf-12* Interaction

A genetic analysis of *daf-12* suggests at least two activities: *daf-12a* promotes third-stage reproductive programs whereas *daf-12b* specifies third-stage diapause (Antebi et al., 1998; Antebi et al., 2000). Presumably these activities are *daf-9* regulated and hormonally specified. Notably, *daf-9* (Daf-c) and *daf-12* (Daf-d) null mutants have opposite phenotypes, implying that hormone might actually inhibit the nuclear receptor with respect to diapause. Moreover, a subset of DAF-12 LBD mutants are Daf-c. A simple hypothesis is that *daf-9* and *daf-12* Daf-c phenotypes result from loss of hormone production and binding, respectively. *daf-12a* and *b* activities could be instructed by presence and absence of a single hormone. Nuclear receptors often activate gene expression in the presence of hormone and repress in their absence. In this view, a *daf-12b(+)* repressor function may be critical for diapause. Alternately, two distinct hormones could specify a and b activities. *daf-9(+)*

Table 2. Adult Life Span

Strain	Temp [°C]	Mean ± SEM [d]	Maximum ± SEM [d]	Mean Last Quartile ± SEM [d]	N[a]
N2	15	25.0 ± 1.8	34.8 ± 1.5	31.3 ± 1.2	519(6)
daf-9(*dh6*)		23.4 ± 1.0*	43.0 ± 5.7	31.4 ± 0.8	175(2)
dh8		22.8 ± 0.8*	42.0 ± 6.4	31.8 ± 1.8	205(3)
dh10		22.7 ± 2.3*	41.0 ± 5.7	30.4 ± 3.6	192(2)
e1406		26.6 ± 0.7	53.0 ± 8.5	38.9 ± 0.6†	77(2)
rh50		20.5 ± 1.3*	33.0 ± 2.8	26.2 ± 1.5	124(2)
daf-12(*rh61rh411*)		22.2*	38.0	28.4	117(1)
daf-16(*mgDf50*)		21.6 ± 1.1*	32.0 ± 4.2	26.3 ± 1.7	238(2)
e1406 rh61rh411		24.1 ± 2.4[d]	37.0 ± 2.8	30.8 ± 1.4†[d]	240(2)
e1406 mgDf50		22.6[d]	56.0	36.9[d]	97(1)
daf-2(*e1368*)		36.8 ± 1.6*	57.0 ± 9.9	46.2 ± 0.6	171(2)
e1370		43.4 ± 3.8*	60.0 ± 5.3	53.6 ± 4.8	281(3)
rh50;*e1368*		29.9 ± 0.2*[b]	46.5 ± 4.9	39.2 ± 2.8	221(2)
rh50;*e1370*		34.1 ± 6.5*[c]	54.0 ± 4.0	46.0 ± 5.8	168(3)
N2	22.5	18.2 ± 0.4	25.5 ± 0.6	22.3 ± 0.6	295(4)
rh50		14.4 ± 0.4*	23.7 ± 0.6	19.6 ± 0.5	248(3)
e1368		29.3 ±0.3*	42.0 ± 1.4	36.7 ± 1.1	185(2)
e1370		28.7 ± 3.5*	47.8 ± 1.0	40.4 ± 1.4	135(4)
rh50;*e1368*		24.9 ± 1.8*[b]	40.5 ± 3.5	30.9 ± 0.1	170(2)
rh50;*e1370*		30.4 ± 6.9[c]	70.5 ± 6.2	50.9 ± 6.6†[c]	159(4)
N2 control	20	21.6 ± 1.4	30.0 ± 1.4		492(4)
Z1–Z4		18.4 ± 1.5*	31.0 ± 2.9		214(4)
Z2–Z3		31.9 ± 2.2*	51.8 ± 1.3		167(4)
rh50 control		20.2 ± 0.9*	32.3 ± 5.9		159(3)
Z1–Z4		17.3 ± 1.1*[e]	30.0 ± 8.5		104(2)
Z2–Z3		18.2 ± 1.8[e]	31.5 ± 0.7		53(2)
dh6;*dhEx24* control		19.7 ± 0.3*	31.5 ± 4.0		253(2)
Z1–Z4		21.3 ± 0.7[f]	37.5 ± 2.6		191(2)
Z2–Z3		26.0 ± 0.9*[f]	42.0 ± 2.3		133(2)
dh6 control		21.2 ± 0.9	45.0 ± 11.3		188(2)
Z1–Z4		25.0 ± 1.0[g]	50.0 ± 9.0		143(2)
Z2–Z3		20.1 ± 1.2[g]	40.5 ± 3.6		213(2)

Significance tests are against N2 unless indicated.
* Significant in T test (<0.0001)
† Significant in Mann-Whitney test (<0.0001). Only applied to last quartile mean when difference in overall mean was not significant.
[a] Number of experiments in parentheses.
[b] Tested against *e1368*.
[c] Tested against *e1370*.
[d] Tested against *e1406*.
[e] Tested against *rh50*.
[f] Tested against *dh6*;*dhEx24*.
[g] Tested against *dh6*.

could synthesize a reproductive hormone, catabolize a dauer hormone, or convert a dauer hormone to reproductive hormone. Generally, *daf-12* mutants with penetrant heterochronic phenotypes have lesions within the LBD, implying hormonal input into the heterochronic circuit as well. However, these mutants have a broader range of heterochronic defects than *daf-9*, e.g., heterochrony in the epidermis, suggesting that other factors may also be involved.

Regulation of Diapause

Based on current genetic and cellular data, we favor the following model for diapause regulation (Figure 6A). Environmental cues (food, pheromone, temperature) transduced by sensory neurons control production of insulin/IGF-I and TGF-β neuroendocrine molecules. In favorable environments, these endocrine factors work through their respective signal transduction pathways to ultimately downregulate DAF-3 and DAF-16. Their downregulation, directly or indirectly, activates the DAF-9 hormone pathway in endocrine tissues. Within cellular targets, hormone triggers DAF-12a transcriptional complexes to promote reproductive and repress dauer programs. In unfavorable environments, either no hormone or an alternate hormone is made. Consequently, DAF-12b complexes specify dauer and repress reproductive programs.

Life Span Regulation

daf-9 mutants display a complex array of life span phenotypes. Maximum life span was increased in all class 2 *daf-9* alleles. However, only *e1406* had a last quartile mean life span significantly longer than that of wild-type at 15°C. Presumably, pleiotropic effects antagonize longevity, as indicated by depression of the overall mean life span. Indeed, class 2 adults exhibit dauer character, including dauer alae, radial constriction, and dark intestines, suggesting that increased longevity could result from heterochronic activation of the dauer program. In *Drosophila*, similar antagonistic effects on life span by

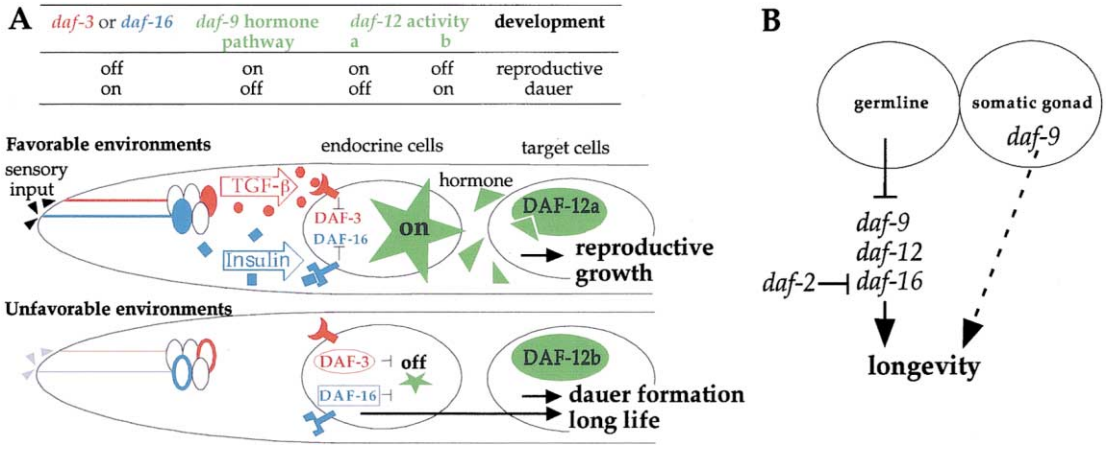

Figure 6. Models for *C. elegans* Life History Regulation (see Discussion)

(A) Regulation of diapause and life span. Endocrine factors TGF-β (red circle) and insulin/IGF-I (blue square), lipophilic hormone (green triangle) and their respective receptors.

(B) Regulation of life span by gonadal signals.

insulin-signaling mutants are also seen, and longevity phenotypes of insulin-like receptor mutants only become apparent in specific allelic combinations (Clancy et al., 2001; Tatar et al., 2001). Jia et al. (2001) saw more dramatic increases in *daf-9* adult longevity than we did. An undiscovered difference between wild-type and mutant strains examined or unidentified difference in culture conditions may be responsible for this.

Furthermore, *daf-9(e1406)* longevity is largely suppressed by *daf-12* but not by *daf-16*, suggesting that *daf-9(+)* inhibits a life-extending activity of *daf-12(+)*. By contrast, *daf-2* longevity is efficiently suppressed by *daf-16* but only weakly suppressed by *daf-12* in some allelic combinations (Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995). This suggests that insulin-like and lipophilic hormonal signals may regulate life span somewhat independently (Figure 6A), although there is probably crosstalk between the pathways. Consistent with this, *daf-9(+)* and *daf-12(+)* promote or antagonize longevity depending on the level or type of activity of the insulin/IGF-I pathway. For example, both *daf-9(rh50)* (this work) and *daf-12* nulls (Gems et al., 1998; Larsen et al., 1995) modestly diminish the longevity of *daf-2* class 1 mutants but greatly enhance the longevity of *daf-2* class 2 mutants.

Finally, we find that, in contrast to wild-type, germline ablations in *daf-9* mutants do not extend life span, either because of direct effects on life span regulation or indirect developmental influences. If direct, then *daf-9* likely acts downstream or parallel to the germline signal. Conceivably, germline signals normally repress *daf-9(+)* and *daf-12(+)* activity (Figure 6B). As a result, longevity genes are silenced. When the germline is ablated, longevity genes are activated by *daf-9(+)* and *daf-12(+)* as well as *daf-16(+)*. Presently, it is unclear whether *daf-9* acts in both germline and somatic gonadal signaling, though *daf-9* expression in the spermatheca is consistent with a function in the latter. It is also unknown whether *daf-16* works independently of *daf-9* and *daf-12*. Paradoxically then, *daf-9(+)* functions to extend life span in the gonadal pathway but shortens life span in the dauer pathway.

Conserved Endocrine Networks

Signaling mechanisms that regulate the rate of aging may have evolved as checkpoints to coordinate environmental conditions with growth and reproduction. Presumably signals from nervous system, gonad, and perhaps other tissues must be integrated to mediate consistent organismal choices. Notably, across nearly all phyla caloric restriction delays reproductive maturity (analogous to diapause) and extends life span (Finch, 1994). In times of food scarcity, it may be evolutionarily adaptive to defer reproduction until conditions improve.

A critical question is whether the networks that control energy homeostasis, reproductive development, and longevity in worms do so in higher organisms. Evidently, insulin/IGF-I signaling regulates reproductive diapause and life span in flies, in part by control of juvenile hormone synthesis (Clancy et al., 2001; Tatar et al., 2001). In mice, a brain-specific knockout of the insulin receptor as well as IRS-2 knockouts reduce fertility by regulating reproductive hormones (Bruning et al., 2000; Burks et al., 2000). Long-lived Ames and Snell dwarf mice, which have mutations in transcription factors of the pituitary, display pleiotropic endocrine deficiencies, including reduced levels of IGF-I (reviewed in Gems and Partridge, 2001). Similarly, long-lived Laron mouse dwarfs, which contain knockouts of growth factor receptor, have reduced serum IGF-I. Recently, a linkage group on chromosome 4 has been associated with exceptional longevity in humans (Puca et al., 2001). It will be interesting to see if elements of the same endocrine networks also determine human life span.

Experimental Procedures

Culture Conditions

Nematode stocks were cultured at 20°C on NG agar seeded with *E. coli* strain OP50 unless indicated otherwise. NG contains cholesterol added to 5 μg/ml. NG minus cholesterol medium omits cholesterol. Cholesterol-deficient medium resembles NG except bactopeptone and cholesterol were omitted, and agarose (SeaKem GTG) replaced agar (Yochem et al., 1999). For this media, OP50 cultures were washed in M9 five to six times before plating. Phenotypes were generally observed in first or second generations.

Mutant Isolation

For mutant screens, N2 cultures were mutagenized with 0.5% EMS in M9 buffer. In noncomplementation screens using *daf-9(rh50) dpy-8(e130)*, 70,000 genomes were examined for F1 transheterozygotes with the Mig phenotype to obtain *dh6*, *dh9*, and *dh10*. In F2 screens, 50,000 genomes were examined for animals with the Mig phenotype to obtain *dh4*. In a clonal screen, 4,200 genomes were examined for Daf-c larvae in the F2 to obtain *dh7*, *dh8*, and *dh11*. Mutants were outcrossed at least three times.

Mutant Rescue

For YAC rescue, total yeast DNA was prepared using the Qiagen Blood and Cell Kit and microinjected (50–100 ng/μl) into *rh50* along with pTG96 *sur-5::gfp* (Yochem et al., 1998) transformation marker (75–100 ng/μl). Cosmids were similarly microinjected individually or pooled (total concentration, 10–20 ng/μl). Two of five Y72A10 lines, three of four Y63B4 lines, and all ten T13C5 lines rescued the gonadal Mig phenotype. Transgenic arrays, *dhEx24* and *dhEx38* (T13C5, TG96), fully rescued the *dh6* Daf-c phenotype.

Molecular Biology

daf-9 cDNAs were obtained by RT-PCR as described (Antebi et al., 2000), using SL1 and gene-specific primers downstream of the predicted stop codon. To obtain the 3′ end, oligo-dT and gene-specific primers were used. Details are available upon request. For *daf-9::gfp* construction, a 9.7 kb genomic fragment containing the *daf-9* coding region and 7.4 kb upstream was amplified with primers 5′-GTAGTAATCT GTCCATTGGG GACTACTG-3′ and 5′-GGGGTAC CTT GATGAGACGA TTTCCGACC-3′, and cloned into pCR-Blunt II-TOPO vector (Invitrogen). *gfp* coding region was then cloned into KpnI-digested *daf-9*-TOPO. *daf-9::gfp* (10 ng/μl) and *lin-15(+)* marker (75 ng/μl) were injected into *lin-15(n765)* animals. *dhEx61* and *dhEx66* extrachromosomal lines fully rescued *daf-9* Mig and Daf-c phenotypes. Integrants *dhIs59* and *dhIs64* were made from these as described (Antebi et al., 2000). IL1/URAVs were identified based on their position relative to established landmarks (White et al., 1986). Occasionally, one of the pair was not ventral, suggesting that other neurons, e.g., IL1D can express *daf-9::gfp.*

Life Span Assays

Adult life span assays were performed as described (Gems et al., 1998). Day 0 corresponds to L4 stage. For *daf-9* Daf-c strains, partial dauer larvae were placed at 15°C or 20°C. After 3–6 days recovered larvae (those that had undergone radial expansion, vulval morphogenesis, and initialized gametogenesis) were taken for adult life span determinations. Statistical analyses were performed with the Excel 98 Student's t test and Mann-Whitney test (Lowry, 2001).

Cell Ablations

Gonad ablations were performed as described (Hsin and Kenyon, 1999), except that L1 larvae were anaesthetized in 0.5% phenoxypropanol in M9 buffer. *rh50* life span was determined in two independent experiments, each at 20°C and 22.5°C. As many as one-third of wild-type and two-thirds of *rh50* Z2, Z3-ablated animals exploded as adults and were excluded from analysis. For *dh6*, two independent experiments were performed at 20°C using *dh6 dhEx24*. Gonadal cells were ablated in L1 larvae, and animals that had recovered to adults were taken for life span analysis. Z1-Z4-ablated animals were judged to be adults based on their increased size, since cellular landmarks are absent. IL1V/URAV ablations were performed on newly hatched L1 larvae of *dh6 dhIs64*. Absence of GFP expression at later stages confirmed cell destruction.

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