Hormonal signals produced by DAF-9/cytochrome P450 regulate *C. elegans* dauer diapause in response to environmental cues

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Summary

In response to the environment, the nematode *C. elegans* must choose between arrest at a long-lived alternate third larval stage, the dauer diapause, or reproductive development. This decision may ultimately be mediated by *daf-9*, a cytochrome P450 related to steroidogenic hydroxylases and its cognate nuclear receptor *daf-12*, implying organism-wide coordination by lipophilic hormones. Accordingly, here we show that *daf-9(+)* works cell non-autonomously to bypass diapause, and promote gonadal outgrowth. Among *daf-9*-expressing cells, the hypodermis is most visibly regulated by environmental inputs, including dietary cholesterol. On in reproductive growth, off in dauer, hypodermal *daf-9* expression is strictly *daf-12* dependent, suggesting feedback regulation. Expressing *daf-9* constitutively in hypodermis rescues dauer phenotypes of *daf-9*, as well as insulin/IGF receptor and TGFβ mutants, revealing that *daf-9* is an important downstream point of control within the dauer circuits. This study illuminates how endocrine networks integrate environmental cues and transduce them into adaptive life history choices.

Key words: Dauer, Hormone, Aging, *C. elegans*, Cholesterol

Introduction

Fundamental insights into how genes and environment influence metazoan metabolism, development and aging have emerged from a genetic dissection of *C. elegans* diapause. Despite its simplicity, *C. elegans* possesses a remarkable endocrine system that regulates choice of arrest at the third larval stage (L3) dauer diapause or continuous reproductive growth, in response to sensory inputs (Cassada and Russell, 1975). Environmental cues favoring dauer include high levels of a crowding pheromone, low nutrient availability and high temperatures presaging imminent starvation or stress (Golden and Riddle, 1984). Arrested before sexual maturity, dauer larvae modify most tissues, shift metabolism, and alter behavior to maximize survival and dispersal. They are stress-resistant, and notably long-lived compared to reproductively growing animals (Larsen, 1993; Lithgow et al., 1995; Murakami and Johnson, 1996; Riddle and Albert, 1997). When conditions improve, they resume development and become reproductive adults with normal life spans, revealing plasticity in the process of aging.

Identified pathways regulating this developmental choice include insulin/IGF, TGFβ, cGMP and serotonergic signaling (Finch and Ruvkun, 2001), which relay neural signals to control programs throughout the body. Insulin/IGF and TGFβ peptides are primary endocrines synthesized and released in response to favorable stimuli, mainly from sensory neurons (Li et al., 2003; Ren et al., 1996). TGFβ signals through its receptors to inactivate DAF-3/SMAD and DAF-5/SNO, allowing reproductive development (da Graca et al., 2003; Estevez et al., 1993; Georgi et al., 1990; Inoue and Thomas, 2000; Patterson et al., 1997; Ren et al., 1996). A complex of DAF-3 and DAF-5 specifies diapause in adverse environments. Insulin/IGF signaling not only controls *C. elegans* dauer, but is also a central regulator of somatic endurance and longevity across taxa (Tatar et al., 2003). Insulin-like agonists stimulate the DAF-2/insulin-like receptor, initiating a kinase cascade that phosphorylates DAF-16 forkhead transcription factor (Kimura 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). This results in cytoplasmic sequestration of DAF-16, and as a consequence animals undergo reproductive growth and live short lives. In adverse environments, DAF-16 enters the nucleus, promoting stress resistance, diapause and longevity (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001).

There is evidence that both insulin/IGF and TGFβ receptors transduce signals through downstream secondary endocrines. Mosaic analysis and tissue-specific promoter studies reveal that *daf-2* regulates diapause and life span by systemic signals (Apfeld and Kenyon, 1998; Wolkow et al., 2000). Similarly, *daf-4*/TGFβ receptor type 2 regulates dauer formation cell non-autonomously (Inoue and Thomas, 2000). As components of nuclear receptor signaling, *daf-9* and *daf-12*, are epistatic to insulin/IGF and TGFβ signaling for diapause regulation, they may comprise this secondary pathway. DAF-9, a cytochrome P450 of the CYP2 class, resembles steroidogenic and fatty acid hydroxylases, as well as xenobiotic detoxifying enzymes (Gerisch et al., 2001; Jia et al., 2002). It probably produces a hormone for DAF-12, a nuclear receptor transcription factor related to vertebrate

vitamin D, pregnane and androstane nuclear receptors (Antebi et al., 2000).

daf-9 mutants fall into two distinct classes (Gerisch et al., 2001; Jia et al., 2002). Strong loss-of-function mutants have dark intestines owing to transient excess fat storage, form dauer larvae constitutively (Daf-c), which eventually recover to sterile adults that live about 25% longer than wild type. Weak loss-of-function mutants have penetrant heterochronic delays in L3 gonadal leader cell migrations (Mig), reduced fecundity and are slightly short-lived. Somewhat opposite, *daf-12* null mutants have impenetrant heterochrony, light intestines, fail to form dauer larvae (Daf-d) and live short lives (Antebi et al., 1998; Gerisch et al., 2001). *daf-9* phenotypes are *daf-12(+)* dependent, showing that *daf-12* acts downstream. Moreover, *daf-9* mutants specifically resemble *daf-12* ligand-binding domain mutants, suggesting that loss of ligand production or binding specify dauer formation. Finally, both *daf-9* and *daf-12* interact with long-lived *daf-2*, enhancing the longevity of strong mutants (class 2) but mildly suppressing longevity of weak mutants (class 1) (Gems et al., 1998; Gerisch et al., 2001). Both are also required for the extended longevity seen in animals whose germline has been ablated (Gerisch et al., 2001; Hsin and Kenyon, 1999), revealing gonadal influences on life span.

A simple model is that DAF-9 produces a hormone for DAF-12, which bypasses diapause, promotes reproductive development and, perhaps, shortens life span. This hormone might be a sterol, as cholesterol deprivation phenocopies larval defects (Gerisch et al., 2001). Expressed in potential endocrine tissues (two head cells, the hypodermis and the hermaphrodite spermathecae), *daf-9* appears to control developmental decisions for the entire organism. However, it is not known whether DAF-9 actually produces hormonal signals, what specific roles the various *daf-9* expressing tissues play, and whether or how *daf-9* is regulated by sensory inputs and upstream signaling pathways. Here we have investigated these issues. Our findings implicate *daf-9* as a central point of developmental control, producing hormonal signals that regulate *C. elegans* life history.

Materials and methods

Nematode culture

Nematodes were cultured at 20°C on *E. coli* strain OP50 on NG agar plates unless indicated otherwise. Transgenes were introduced into different genetic backgrounds by standard crosses. In most *daf-9::gfp* expression experiments, two L4 animals were placed on plates, and their F1 progeny scored for *daf-9* expression levels. Experiments were repeated at least twice. For temperature experiments, animals were scored after two generations of growth at the appropriate temperature, except when arrested as dauer larvae. Preparation and culture on NG agar minus cholesterol and NG agarose minus cholesterol were carried out as described (Gerisch et al., 2001). For food experiments, 2% DH5α *E. coli* growth arrested with streptomycin (50 μg/ml) were used, varying the dilution and volume plated. Dauer pheromone was prepared as described (Golden and Riddle, 1984) and different amounts applied to plates containing 20 μl of 2% DH5α. 2-3 days later, plates were scored for *daf-9::gfp* and dauer formation.

Expression mosaics

Larvae from *daf-9*(*dh6) dhEx66* mothers were examined by fluorescence microscopy for *daf-9* expression with M2-Bio GFP-Binocular (Kramer Scientific), and confirmed at higher magnification with an Axioskop 2 (Zeiss). Scored as reproductive (light intestine, vulval, seam and gonadal cell divisions), or dauer (dauer alae, dark intestine, arrested development, thin body and pharynx), animals were followed to confirm expression pattern and development. Mosaic animals were found at a frequency of ~1/250.

Genotypic mosaics

ncl-1(e1865) was injected with a cocktail of *ncl-1* (cosmid C33C3, 10 ng/μl), 7.1 kb *daf-9::gfp* (10 ng/ μl) and *sur-5::gfp* (pTG96, 75 ng/μl) constructs. Stable F2 extrachromosomal lines (e.g. *dhEx107)* were inspected to verify *ncl-1* rescue and co-segregation of *ncl-1(+)* and *sur-5::gfp* neurons. Mosaics were analyzed from strain *daf-9(dh6) ncl-1(e1865) dhEx107*. L2-L4 animals were initially screened for loss of intestinal or neural *gfp* expression to see P- and AB- mosaics respectively. To determine where in the cell lineage mosaic loss occurred, the following cells were typically scored: CANL/R, ADL/R, BAGL/R, ASKL/R, NSML/R, BDUL/R, ALML/R, ASIL/R, HSNL/R, EXC, Ex gland L/R, Hyp 8, 9, 10, PLML/R, seams, posterior/anterior pharynx, somatic gonad, sex myoblasts, body muscle, intestine, hyp7, vulva and ventral cord neurons (Hedgecock and Herman, 1995). Mosaic animals were found at a frequency of ~1/1000. The array *dhEx24* (T13C5, pTG96) is described elsewhere (Gerisch et al., 2001).

daf-9::gfp expression constructs

daf-9 constructs were made by standard molecular techniques using genomic fragments or isoform B cDNA amplified with gene-specific primers and cloned in front of *gfp*. *daf-9::gfp* contains 7.16 kb upstream promoter and the entire genomic *daf-9*-coding region, including introns with *gfp* fused at the C terminus. This construct was used to make integrant *dhIs59*, *dhIs64* and extrachromosomal *dhEx66*, as previously described (Gerisch et al., 2001). *dhEx67* construct is similar, but has 1.82 kb promoter (forward, CTCCAGTTTTG-GGTGTTTCAGAGCAGCG). Array *dhEx203* was made from the *dr434* construct (Jia et al., 2002), which consists of 3.03 kb of *daf-9* promoter, isoform B cDNA with *gfp* fused at the C terminus. *dhEx82* construct contains 1.15 kb promoter, with intron 1 inserted in *daf-9B* cDNA and *gfp* fused at the C terminus. To make this, a 1.68 kb genomic fragment was PCR amplified (forward, GCTCTAGAGATA-CACCAGGGTATCACTTC; reverse, TCGATTAAGAACATCAGT-TC) and cloned into *Xba*I/*Xho*I sites at the 5´ end of *daf-9* cDNA. *dhEx94* construct contains 1.19 kb *daf-9* promoter, exon and intron 1. A 2.07 kb fragment was PCR amplified (forward, CTCCAGT-TTTGGGTGTTTCAGAGCAGCG; reverse, CCCGGTACCTGAT-CTGAAATTTTAATATT), digested with *Sal*I/*Kp*nI and a 1.44 kb subfragment cloned into L3781 (A. Fire, personal communication).

dhEx300 construct contains 0.27 kb promoter (forward, TGTT-GCAAATGTTCAAAATGTCACGCTCA; reverse, GCGGTACCAT-TACGAGTGGCATACTGTAT), fused directly to *gfp*.

Heterologous tissue-specific constructs were made by inserting amplified fragments into *Pst*I/*Bam*HI sites in front of *daf-9* cDNA, with *gfp* fused at the C terminus, using the following promoter regions: 0.64 kb *col-3* (*dhEx207*), 1.15 kb *dpy-7* (*dhEx217)*, 3.45 kb F25B3.3 (*dhEx256*), 0.63 kb *mec-7 (dhEx176*), 3.59 kb *sdf-9 (dhEx354)* and 3.18 kb *wrt-1* (*dhEx294*). The following primers carrying restriction sites for *Pst*I (F primer) and *Bam*HI (R primer) were used: *col-3* (forward, GCCTGCAGCTACTTCTACACACAT-TGCAA; reverse, GCGGATCCGTTGGAAACTGAAGATTCTCA); *dpy-7* (forward, GCCTGCAGCTATGTGCAATGTCACGTGGA; reverse, GCGGATCCCTGGAACAAAATGTAAGAATA); F25B3.3 (forward, GACTCTGCTGCAGAAAATATCTCGTCATC; reverse, GCGGATCCGATATTCTGAACAAGAAACCA); *mec-7* (forward, GCCTGCAGGAGCTACGCCGAACTTGGAG; reverse, GCGGA-TCCGACGAATAATGGAGGAGTCA); *sdf-9* (forward, GCCTGC-AGGTCGACTTGTCAATGTCGCAG; reverse, GCGGATCCTTTG-AAAATAATATATCTAGT); *wrt-1* (forward, GCAAGCTTGTGCAA-

GCACAGCTAGAGGTC; reverse, GCGGATCCCATCGGATTGTG-ATTAGCTTC).

For F25B3.3, an internal *Pst*I site was used for cloning. To express *daf-9* under the *wrt-1* promotor we introduced a *Hin*dIII site into the F-primer. A 0.12 kb *Hin*dIII/*Bam*HI fragment of *wrt-1* was cloned infront of the *daf-9* cDNA, then a *Hin*dIII fragment of 3.04 kb was added. Transgenic animals were made by injecting constructs at a concentration of 10-20 ng/μl with *lin-15(+)* marker plasmid at 75-90 ng/μl into the germline of *lin-15(n765)* animals.

Quantitation of *daf-9* expression

gfp fluorescence of *dhIs64* and *dhIs59* animals grown at different temperatures was imaged through an Axioplan 2 Microscope (Zeiss) and photographed with a Hamamatsu ORCA-ER camera. Pixel intensity over a fixed area was measured with Axiovision 3.1 software (Zeiss). These measurements were then used as a reference to quantitate expression in other genotypes or culture conditions.

Results

daf-9 acts cell non-autonomously

An integrated genomic *daf-9::gfp* construct, *dhIs64*, rescues *daf-9* dauer constitutive (Daf-c) and cell migration (Mig) phenotypes (Gerisch et al., 2001). *daf-9*-expressing tissues include a bilateral ventral pair of head cells in the anterior ganglion, identified as XXXL/R (Ohkura et al., 2003), the syncitial epidermal tissue surrounding the worm called the hypodermis and the hermaphrodite spermathecae. Although the XXXL/R cells are described as embryonic hypodermal cells, they later appear to have neuronal character including a small pocked nucleus in larvae and adults, as well as axon-like processes in dauer larvae. *daf-9* expression in XXX cells appears from late embryos to old adults, and is upregulated in dauer larvae. Hypodermal *daf-9* is dramatically regulated in an all or none fashion during larval development. Expression begins at mid-L2, the time of commitment to reproductive development, and is downregulated in L4. In the dauer stage, hypodermal *daf-9* is not expressed. Spermathecal expression begins in late L4 and continues in adults. Therefore our attention was drawn to hypodermis and XXX cells as important for dauer regulation.

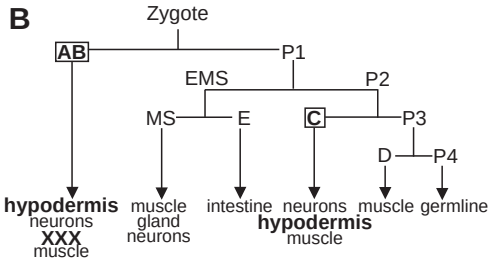
To understand how *daf-9*-expressing tissues impact gene function, we asked what phenotypes were restored when *daf-9* was selectively expressed. Initially, we generated expression mosaics: transgenic lines containing an extrachromosomal array of genomic *daf-9::gfp* (*dhEx66*) in the *daf-9(dh6)* background. Animals expressing *daf-9* in hypodermis and XXX cells reached maturity, whereas cohorts without the transgene arrested as dauer larvae. Mosaics arise spontaneously either from mitotic loss or silenced expression of the array in a particular tissue. At both 20°C and 25°C, mosaics expressing *daf-9* in the hypodermis bypassed diapause, had normal gonadal development and were fertile (Fig. 1A). This indicates that hypodermal expression is sufficient to drive reproductive development, including the correct timing of distal tip cell migrations. Interestingly, in such mosaics hypodermal *daf-9* expression levels were often elevated, implying that *daf-9*-expressing XXX cells normally communicate cell non-autonomously to inhibit expression in the hypodermis. Mosaics expressing *daf-9* in XXX cells alone were not found, probably because of their rarity.

Genotypic mosaics, where mitotic loss of *daf-9::gfp* was followed by linked cell autonomous markers *sur-5::gfp* and *ncl-1(+)* (Hedgecock and Herman, 1995; Yochem et al., 1998) in extrachromosomal array *dhEx107*, supported the above findings. Of the first two blastomeres, AB generates nearly all neurons, XXX cells, hypodermis and anterior pharynx, while P1 gives rise to germline, muscle, intestine, gonad, hypodermis, posterior pharynx and mostly pharyngeal neurons (Fig. 1B) (Sulston et al., 1983). When *daf-9(+)* was present in both blastomeres (AB+P+), animals always reached maturity, whereas AB-P- animals arrested as dauer larvae (Fig. 1C). AB-P+ mosaics, which do not express *daf-9* in XXX cells reached adult, again implying that expression of *daf-9* in XXX cells is not absolutely required for reproductive growth. Similarly, loss from P1 as well as from P1-derived blastomeres

A ***dh6 dhEx66***

Temp	H	X	Growth	Broods
20°	+	+	Rep (*n*>100)	238 ± 18 (*n*=6)
	-	-	Daf-c (*n*>100)	0 (*n*>100)
	+	-	Rep (*n*=20)	253 ± 106 (*n*=5)
25°	+	+	Rep (*n*>100)	50 ± 21 (*n*=8)
	-	-	Daf-c (*n*>100)	0 (*n*>100)
	+	-	Rep (*n*=27)	41 ± 33 (*n*=17)

B



C ***dh6 ncl-1(e1865) dhEx107***

Genotype	Growth
AB+ P1+	Rep (*n*>100)
AB- P1-	Daf-c (*n*>100)
AB- P1+	Rep (*n*=3)
AB- EMS- P2+	Rep (*n*=1)
AB- EMS- C+ D-	Rep (*n*=2)
AB+ P1-	Rep (*n*=3)
AB+ EMS- P2+	Rep (*n*=2)
AB+ EMS- C+ P3-	Rep (*n*=1)
AB+ MS+ E- P2+	Rep (*n*=3)

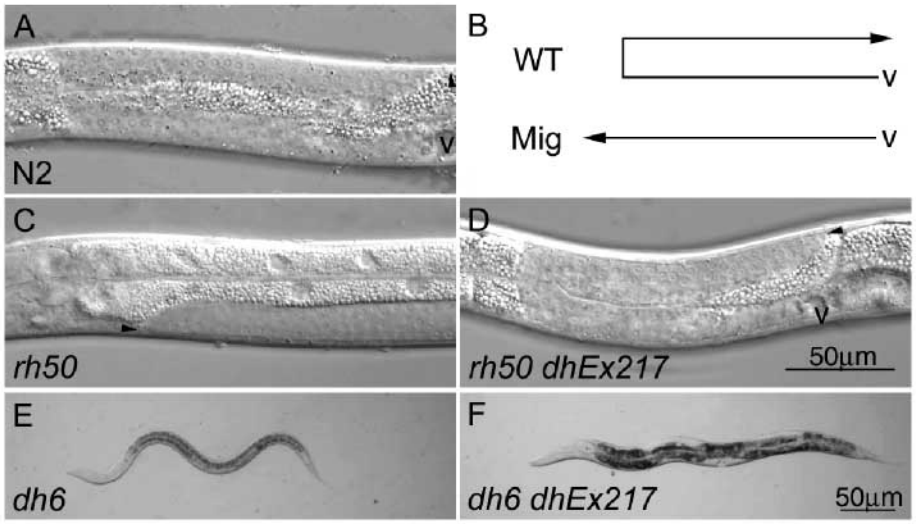
D ***dh6 dhEx24***

Genotype	Growth
AB+ P1+	Rep (*n*>100)
AB- P1-	Daf-c (*n*>100)
AB- EMS- P2+	Rep (*n*=2)
AB- EMS+ P2-	Daf-c (*n*=1)
AB- EMS- C+ P3-	Rep (*n*=2)
AB- EMS- C- D+ P4-	Daf-c (*n*=1)
AB+ P1-	Rep (*n*=2)
AB+ EMS- P2+	Rep (*n*=1)
AB+ MS+ E- P2+	Rep (*n*=2)

Fig. 1. Mosaic analysis. (A) *daf-9*(*dh6*) *dhEx66* [*daf-9::gfp, lin-15(+)*] expression mosaics. Loss (–) or retention (+) of expression from hypodermis (H) or XXX cells (X), and effects on mode of growth (Daf-c, Reproductive) and broods. (B) Fate map of the early blastomeres. XXXL/R arise from AB, hypodermis from AB and C (bold). Genotypic mosaics of *dhEx107* [*daf-9::gfp, sur-5::gfp, ncl-1(+)*] in *daf-9(dh6) ncl-1(e1865)* (C), and *dhEx24* [T13C5, *sur-5::gfp*] in *daf-9(dh6)* (D). *daf-9*(+) or (–) genotypes of the blastomeres are indicated beneath, with phenotypic mode of growth.

resulted in reproductive adults. Presumably, *daf-9* function is provided by hypodermal cells, which originate in both AB and P1 lineages. Consistent with this, we found rare mosaics (Fig. 1C, *n*=2) in which *dhEx107* was retained solely in the C blastomere (predominately hypodermis, muscle, and two neurons) that reached adult. Again, mosaics expressing *daf-9* in XXX cells only were not found.

One concern is that *daf-9::gfp* was overexpressed in extrachromosomal arrays, owing to multiple copies of the gene. We obtained similar results when *daf-9* was present at an estimated fivefold lower molar ratio using the *daf-9* cosmid T13C5 in an array marked with *sur-5::gfp* (*dhEx24*). Mosaics in which *daf-9(+)* was included in P2 or C only, but absent from other blastomeres reached reproductive maturity (Fig. 1D, *n*=4). In summary, we conclude that *daf-9* works cell non-autonomously, and that hypodermal *daf-9* can be sufficient to promote reproductive growth. Thus, the hypodermis is a major endocrine tissue that regulates development.



H	X	S	Rescue	Name
+	+	+	yes	*dhEx66, dhIs64*
+	+	+	yes	*dhEx67*
-	+	-	partial	*dhEx203*
+	+	+	yes	*dhEx82*
+	+	+	n.d.	*dhEx94*
-	+	-	n.d.	*dhEx300*

Fig. 2. Constitutive hypodermal overexpression rescues *daf-9* larval phenotypes. (A) N2 wild type, gonadal distal tip cells (arrowhead) turn normally, as diagrammed in B. (C) *daf-9(rh50)*, distal tip cells fail to turn and remain on the ventral body wall (Mig), shown in B. (D) *dhEx217* [*pdpy-7::daf-9*] transgene rescues *rh50* Mig phenotypes. (E) *daf-9(dh6)* Daf-c dauer larva. (F) *dhEx217* transgene rescues *dh6* Daf-c phenotypes. v, vulva. (G) *daf-9::gfp* fusion constructs. Expression in hypodermis (H), XXX cells (X) and spermatheca (S), and rescue activity in *daf-9(dh6)* or *daf-9*(*e1406*).

Tissue-specific rescue of *daf-9* phenotypes

Hypodermal *daf-9* expression

As an alternative to mosaics, we made *gfp* fusions to *daf-9* cDNA driven by tissue-specific promoters. When fused to promoters from *col-3* (*dhEx207*, hypodermis, seam cells, P ectoblasts) (Cox and Hirsh, 1985), *dpy-7* (*dhEx217*, hypodermis, seam cells) (Gilleard et al., 1997) and *wrt-1* (*dhEx294*, hypodermis) (Aspock et al., 1999), near wild-type function was restored in *daf-9(dh6)* and *daf-9(rh50)* (Fig. 2, Table 1). Transgenically rescued animals bypassed diapause, had normal light intestines, complete gonadal reflexion and large broods, further demonstrating that *daf-9* functions cell non-autonomously and that hypodermal *daf-9* suffices for reproductive growth. Active from embryo (*dpy-7*) and early larvae (*col-3*) to adult, and from embryo to L4 (*wrt-1*), these promoters expressed hypodermal *daf-9* earlier and later than normal with no obvious effect, except that *pdpy-7::daf-9* transgenics were Daf-d, failing to form dauer larvae on starved out plates (*n*>300). Thus, constitutive hypodermal expression resulted in a gain-of-function (Daf-d) opposite the loss-of-function phenotype (Daf-c).

pdpy-7::daf-9 had minor effects on one *daf-12* ligand binding domain mutant: the *daf-12(rh273)* Daf-c phenotype was suppressed, while the gonadal Mig phenotypes of *rh273* and *rh61* were not (Table 1). The missense allele *rh273* probably reduces but does not abolish activity of the ligand binding domain, whereas *rh61* truncates the receptor and probably abrogates ligand binding altogether. Irrespective of the actual molecular behavior of these mutant proteins, failure to suppress *daf-12* Mig phenotypes place *daf-9* upstream of the *daf-12* ligand binding domain.

Epistasis experiments with *daf-9* loss-of-function place it downstream or parallel to TGFβ and insulin/IGF signaling (Gerisch et al., 2001; Jia et al., 2002). We asked how constitutive hypodermal overexpression affects these pathways. We found that *pcol-3::daf-9* and *pdpy-7::daf-9* potently suppressed Daf-c phenotypes of null allele *daf-7(m62)*/TGFβ and partially restored progeny production (Table 2). However, dark intestine and egg laying defects were unaffected. Similarly, these transgenes suppressed the Daf-c phenotype of insulin receptor mutant *daf-2(e1368)* and partially restored progeny production. A stronger allele, *daf-2(e1370)* was also suppressed for dauer morphogenesis, but animals arrested as sterile, dark L3/L4 larvae. Delayed or arrested cellular development was evident in most tissues, including somatic gonad, germline, seam and vulva. In addition, the *pcol-3::daf-9* transgene also had no effect on *e1368* and *e1370* longevity (data not shown). Thus, constitutive hypodermal *daf-9* overexpression only partly rescues *daf-2* for diapause.

Table 1. Phenotypes of tissue-specific expressed *daf-9cDNA::gfp* constructs in *daf-9* and *daf-12* backgrounds

Genotype	Construct	Promoter	Expression	% Mig	% Daf-c	Broods
N2	–	–	–	0	0	143±49
	dhIs64	*daf-9*	X, H, S	0	0	26±31
	dhIs59	*daf-9*	X, H, S	0	0	37±24
	dhEx217	*dpy-7*	H	0	0	93±20
	dhEx203	*daf-9*	X	0	0	100±25
daf-9(dh6)	–	–	–	0	100	0
	dhIs64	*daf-9*	X, H, S	0	0	35±28
	dhEx217	*dpy-7*	H	0	0	77±12
	dhEx176	*mec-7*	Touch N	nd	100*	0*
daf-9(e1406)	–	–	–	0	100	0
	dhEx294	*wrt-1*	H	2	0	73±49
	dhEx256	F25B3.3	Pan N	8, 23†	40*, 61	17±22
	dhEx203	*daf-9*	X	4, 15†	0*, 38	21±21
	dhEx354	*sdf-9*	X	2	0	107±76
daf-9(rh50)	–	–	–	79	0	32±19
	dhEx217	*dpy-7*	H	0	0	80±37
daf-12(rh273)	–	–	–	70	59	14±6
	dhEx217	*dpy-7*	H	42	0	3±5
daf-12(rh61)	–	–	–	100	0	23±10
	dhEx217	*dpy-7*	H	74	0	17±14

Mig phenotype (20°C) $n \geq 25$ animals. Daf-c phenotype (25°C) $n \geq 150$ animals. Broods (25°C) $n \geq 6$ animals. X, XXX cells; H, hypodermis; S, spermathecae; N, neuron.

*20°C.

†25°C.

Table 2. Phenotypes of tissue-specific expressed *daf-9cDNA::gfp* constructs in *daf-2* and *daf-7* backgrounds.

Genotype	Construct	Promoter	Expression	% Daf-c	Broods
N2	–	–	–	0	143±49
daf-2(e1368)	–	–	–	100	0
	dhEx207	*col-3*	H	0 (n=43)	n.d.
	dhEx217	*dpy-7*	H	0	39±15
	dhEx256	F25B3.3	Pan N	95	70±28*
daf-2(e1370)	–	–	–	100	0
	dhEx379‡	–	–	100	0
	dhEx207	*col-3*	H	0† (n=22)	n.d.
	dhEx217	*dpy-7*	H	0†	0
	dhEx354	*sdf-9*	X	100 (n=50)	0
	dhEx256	F25B3.3	Pan N	100	0
daf-7(m62)	–	–	–	100	0
	dhEx207	*col-3*	H	6	n.d.
	dhEx217	*dpy-7*	H	0	52±23
daf-7(e1372)	–	–	–	100	0
	dhEx354	*sdf-9*	X	100 (n=50)	0

*Brood size of reproductively growing animals

†Arrest development at L3 or early L4 stages

‡*lin-15(+)* transgene alone.

Daf-c and Brood size experiments were carried out at 25°C. Daf-c phenotype, $n \geq 150$ animals, unless indicated. Brood size, $n \geq 8$ animals.

daf-9 expression in XXX cells and neurons

As originally observed by Jia et al., *daf-9* cDNA driven by 3 kb of endogenous upstream promoter expressed strongly only in XXX cells (Fig. 2G), and not in hypodermis (Jia et al., 2002). At 20°C, an array containing this construct (*dhEx203*) fully rescued the Daf-c phenotype of *daf-9(e1406)*, but a small fraction (4%) were Mig, indicating near complete rescue (Table 1). At 25°C rescue was less efficient as many were Daf-c (38%) or Mig (15%). *sdf-9*/phosphatase is also expressed in the XXX cells (Ohkura et al., 2003), and *daf-9* expression under this promoter actually gave more complete rescue at 25°C. We conclude that *daf-9* in the XXX cells regulates diapause cell non-autonomously, and provides at least partial activity to promote reproductive development. By contrast, *daf-9* expression in XXX could not rescue the Daf-c phenotypes of *daf-2* and *daf-7* (Table 2).

daf-9 is also weakly expressed in a few unidentified neurons (Gerisch et al., 2001; Jia et al., 2002). Pan-neuronal expression using the F25B3.3 promoter (*dhEx256*) (Altun-Gultekin et al., 2001) also rescued Daf-c and Mig phenotypes, but much less effectively than XXX expression (Table 1). Finally, expression in touch neurons with the *mec-7* promoter (Hamelin et al., 1992) failed to rescue (*dhEx176*, Table 1), revealing that *daf-9* must be expressed in an appropriate subset of neurons.

daf-9 promoter constructs

To define the *daf-9* promoter regions mediating tissue-specific expression, we generated a number of constructs (Fig. 2G). A 1.44 kb fragment containing 1.19 kb of promoter, exon and intron 1 maintained expression in all tissues (*dhEx94*). 0.27 kb promoter fused directly to *gfp*, maintains robust XXX cell expression only (*dhEx300*), revealing that an XXX element resides in this small region, while hypodermal and spermathecal elements may lie upstream and downstream of this. Indeed, the *daf-9* cDNA construct with 3.03 kb of promoter but lacking introns (*dhEx203*) was expressed solely in XXXL/R, and reintroducing intron 1 restored hypodermal and spermathecal expression (*dhEx82*). Thus, their elements probably reside within intron 1.

Environmental influences on *daf-9* expression

We next looked at the effect of environmental conditions on *daf-9* expression under control of the endogenous promoter. We varied temperature, cholesterol, food and dauer pheromone, and observed *daf-9::gfp* levels by fluorescence

microscopy. Integrants, *dhIs64* as well as *dhIs59*, which expressed at half the level (data not shown), gave similar results. Hypodermal *daf-9* showed a striking pattern of regulation by environmental conditions, as follows. In favorable environments (abundant food and cholesterol, 20°C, low pheromone) hypodermal *daf-9* was weakly expressed (Figs 3, 4). Conditions of mild stress (reduced food and cholesterol, 22-25°C, higher pheromone) not sufficient to drive dauer formation, resulted in upregulation. However, in strongly dauer inducing conditions (low food and cholesterol, 27°C, high pheromone) hypodermal *daf-9* was switched off, as detailed below.

Temperature

In wild type, higher temperatures favor dauer formation, and at 27°C typically about 5-20% of a culture form dauer larvae despite low population density and abundant food (Ailion and Thomas, 2000). Hypodermal *daf-9* was visibly sensitive to temperature (Fig. 3). Weakly expressed at 15°C and 20°C, it was upregulated about eightfold at 22.5°C, and at least 13-fold at 25°C. At 27°C, reproductively growing animals had high hypodermal expression while those entering dauer had none (Fig. 3E,H). By contrast, expression in XXX was constant in L3 at most temperatures, but upregulated in dauer larvae formed at 27°C (Fig. 3F,G,I). In 2-day-old adults, XXX expression varied inversely with temperature (Fig. 3J), while *daf-9* spermathecal expression was unaffected by temperature (Fig. 3K).

Cholesterol

C. elegans requires cholesterol for growth and development. Wild-type animals cultured in cholesterol-deficient media display gonadal Mig phenotypes similar to *daf-9(rh50)* and form dauer-like larvae (Gerisch et al., 2001). We observed that hypodermal *daf-9* was also sensitive to dietary cholesterol (Fig. 4A-D). A minor reduction (NG minus cholesterol plates and washed OP50), as judged by absence of cellular phenotype, maximally upregulated hypodermal *daf-9*. A further reduction (agarose plates with washed OP50), as judged by presence of Mig animals, did not lead to a further increase. Finally, in dauer larvae formed by cholesterol deprivation there was no expression. Excess cholesterol had no effect.

Food

In abundant food, hypodermal *daf-9* was weakly expressed. As food is decreased 10-fold, expression increased (Fig. 4E). Further dilution led to weaker or no expression. Some of these animals formed dauer larvae.

Pheromone

As pheromone increased, hypodermal *daf-9* increased in expression (Fig. 4F). At higher pheromone concentrations, when animals entered dauer, expression ceased.

Taken together, these results imply that the response of hypodermal *daf-9* to environmental cues modulates dauer commitment, antagonizing it under weak dauer-inducing conditions.

Genetic influences on *daf-9* expression

We next examined the effect of genotype by introducing *daf-9::gfp* (*dhIs64*) into various Daf mutant backgrounds

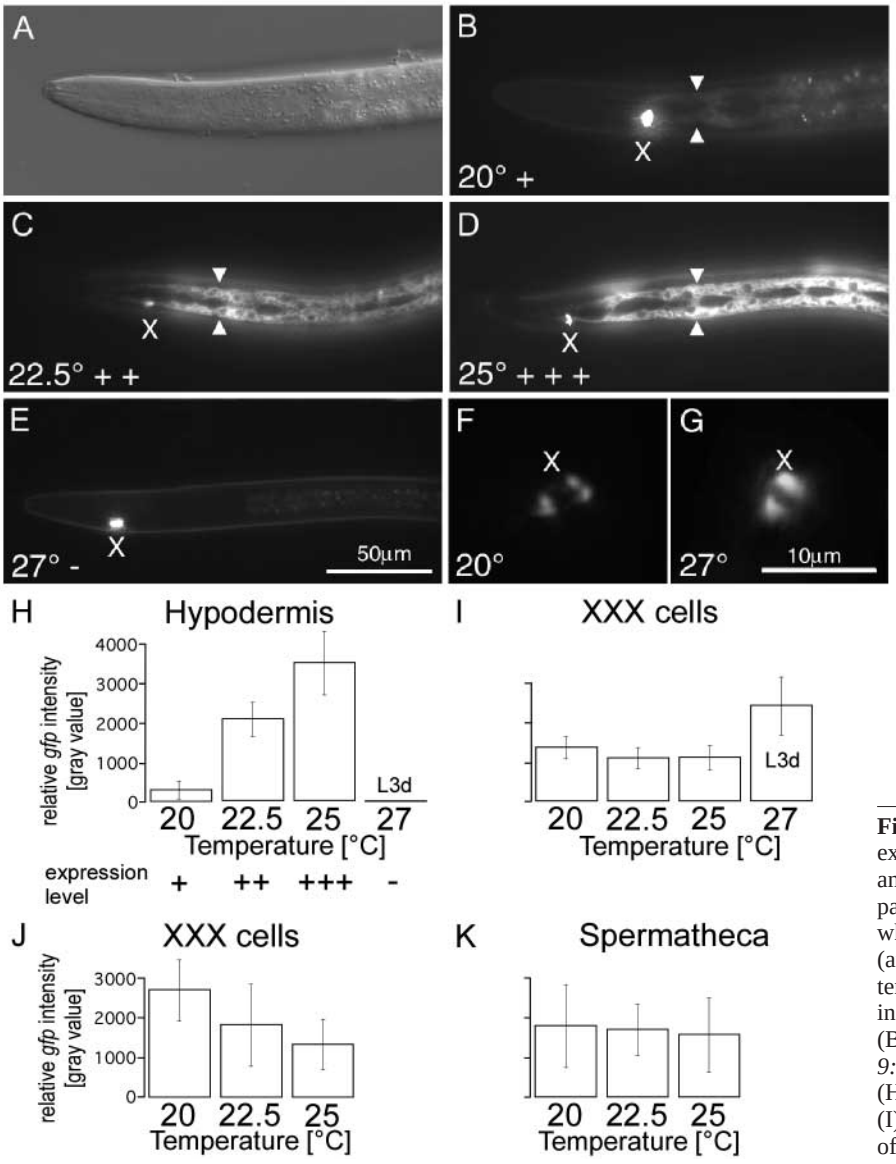


Fig. 3. Thermal influences on N2 *dhIs64* expression. (A) DIC micrograph of head region of an L3 larva. (B-G) *gfp* fluorescent images. All panels are reproductive L3 larvae, except E and G, which show a dauer larva. Hypodermis (arrowheads) and XXX cells (X). Growth temperature and expression level (–, +, ++, +++) are indicated. C-E) are taken at 200 ms exposure time, (B) at 800 ms. (H-K) Quantitation of *daf-9::gfp(dhIs64)* intensity measurements. (H) Hypodermis of L3 and L3d (dauer) as well as (I) XXX cells. (J) XXX cells and (K) spermathecae of 2-day-old adults.

representing nuclear hormone, insulin/IGF, TGFβ and cGMP signaling (Table 3). Similar results were obtained with *dhIs59* (Table 4).

Nuclear hormone signaling

When we crossed *daf-9::gfp* into different *daf-12* mutants, we saw striking changes in hypodermal *daf-9* expression. First, *daf-12* null allele *rh61rh411*, which is Daf-d, failed to express hypodermal *daf-9* but had no effect on expression in other tissues (Table 3, Fig. 4D, Fig. 5D). Similarly, *daf-12(sa156)*, a Daf-d missense mutation in the DNA-binding domain that abrogates transcriptional activation (Shostak, 2002) abolished hypodermal expression. In fact, all perturbations that upregulated hypodermal *daf-9* (25°C, low cholesterol, low food, high pheromone) had no effect in the *daf-12* null background. We conclude DAF-12 transcriptional activity, directly or indirectly, promotes hypodermal synthesis in response to environmental inputs. This is surprising as epistasis experiments place *daf-12* downstream of *daf-9* (Gerisch et al., 2001; Jia et al., 2002). Therefore, *daf-12* probably regulates *daf-9* via a feedback loop.

daf-12 ligand-binding domain mutant *rh273* exhibited a more complex pattern. As mentioned above, *rh273* affects a predicted ligand contact site and may reduce affinity for hormone (Antebi et al., 2000). Some animals arrest as Daf-c dauer larvae, whereas the remainder resume development but are Mig. Whereas animals that had bypassed diapause overexpressed hypodermal *daf-9*, those arrested as dauer larvae did not express at all (Fig. 5E,F). Similar behavior was seen with *rh274*, which affects the same residue (data not shown). Apparently, reduction of ligand binding can lead either to an abrupt increase or decrease of hypodermal expression, suggesting that these mutants lie at the cusp of the dauer decision.

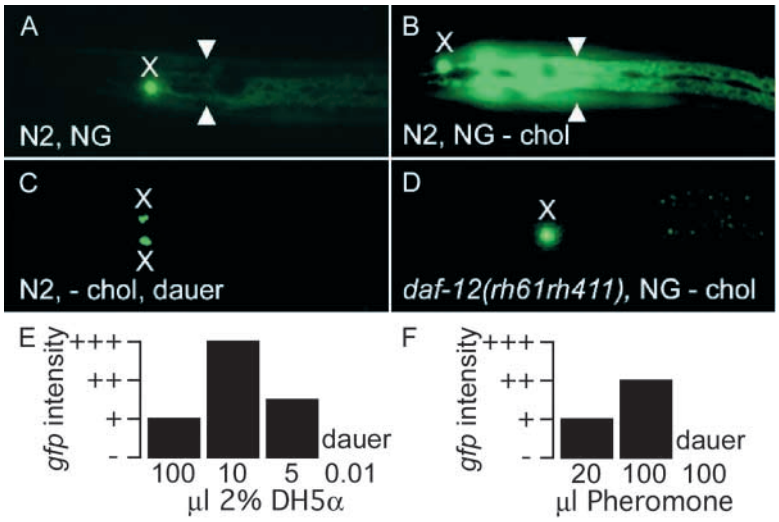


Fig. 4. Influence of cholesterol, food, and pheromone on *daf-9* expression of L3. (A) N2 *dhIs64* grown on normal nematode growth (NG) media. (B) N2 *dhIs64* grown on NG lacking added cholesterol (NG – chol). (C) N2 *dhIs64* dauer larva grown on NG agarose lacking cholesterol (– chol). (D) *daf-12(rh61rh411) dhIs64* grown on NG lacking cholesterol. (E) Hypodermal *daf-9* expression with increasing dilutions of 2% DH5α. (F) Hypodermal *daf-9* expression at different concentrations of pheromone. Hypodermis (arrowheads) and XXXL/R cells (X). *daf-9::gfp* intensity (–, +, ++, +++), see Fig. 3. Worms are cultured at 20°C.

We reasoned that if reduced hormone binding upregulates hypodermal *daf-9*, then *daf-9* mutants themselves, presumably diminished in hormone production, could also influence hypodermal expression. We made a promoter fusion consisting of 1.19 kb upstream region plus the first intron of *daf-9* joined to *gfp* (*dhEx94*) (Fig. 2G). Expressed in all three cell types, this transgene makes no functional DAF-9 product. When introduced into the hypomorphic mutant *daf-9(rh50)*, *dhEx94* was strongly overexpressed (Fig. 5B), suggesting autoregulation. By contrast, in the *daf-9(dh6)* null background, hypodermal expression was off (Fig. 5C). Thus, although partial reduction of *daf-9* activity stimulates hypodermal *daf-9* expression, complete loss of *daf-9* activity does not.

Insulin/IGF signaling

We crossed *daf-9::gfp* into the backgrounds of insulin/IGF pathway mutants *daf-2*/insulin/IGF receptor and *daf-*

Table 3. Genotypic influences on *daf-9::gfp (dhIs64)* hypodermal *daf-9* expression

	20°C		25°C	
Genotype	L3	L3d	L3	L3d
N2	+		+++	
daf-9(dh6)	+		+++	
daf-12(rh61rh411)	–		–	
sa156	–		–	
rh61	–		+	
rh273	+++	–	+++	–
daf-2(e1368)	+++			–
e1370	+++	–		–
daf-16(mgDf50)	+		+++	
daf-7(e1372)	+++	–		–
daf-3(mgDf90)	+		+++	
daf-5(e1386)	+		+++	
daf-11(m47)	+++	–	+++	–
osm-6(p811)	+		+++	–
mgDf50 mgDf90	+		+++	
rh61rh411 e1368	–		–	
rh61rh411 e1370	–		–	
rh61rh411 e1372	–		–	
e1370 mgDf50	+++		++++	
e1372 e1386	++		+++	

–, no expression. +, ++, and +++ relative intensity of *daf-9H* expression seen at 20°C, 22.5°C and 25°C, respectively (see Fig. 3).

Average of $n \geq 25$ animals. L3 reproductively growing larvae, L3d constitutive dauer larvae.

Table 4. Genotypic influences on *daf-9::gfp* (*dhIs59*) hypodermal *daf-9* expression

	20°C		25°C	
Genotype	L3	L3d	L3	L3d
N2	–		++	
daf-12(rh61rh411)	–		–	
daf-2(e1370)	+++			–
daf-16(mgDf50)	–		++	
daf-7(e1372)	+++			–

For explanations see Table 3.

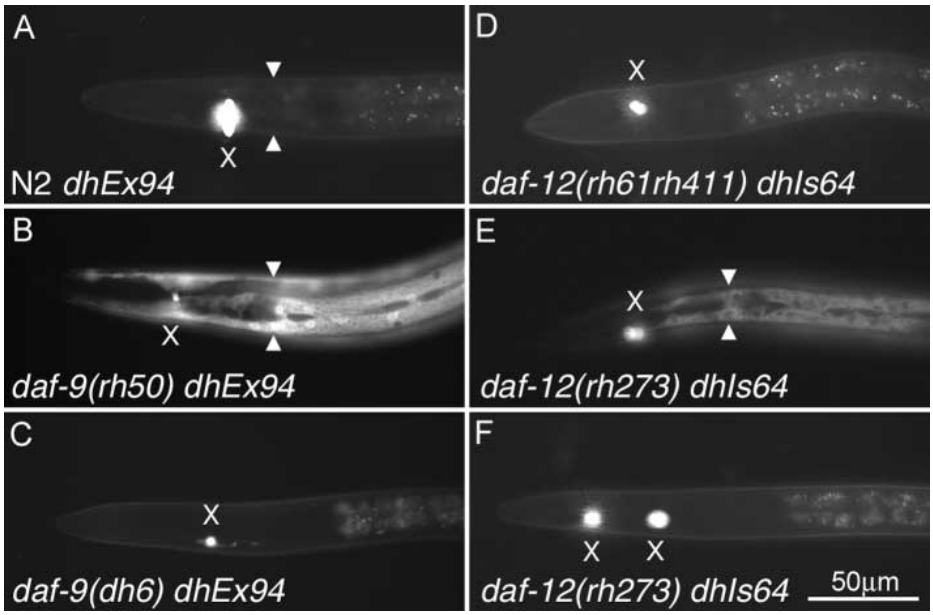


Fig. 5. Hypodermal *daf-9* expression depends on *daf-9* and *daf-12*. (A) N2 *dhEx94*, hypodermal *daf-9* is weakly expressed. (B) *daf-9(rh50) dhEx94*, hypodermal *daf-9* is overexpressed. (C) *daf-9(dh6) dhEx94*, Daf-c dauer larva, hypodermal *daf-9* is not expressed. (D) *daf-12(rh61rh411) dhIs64* at 25°C, *daf-9* is expressed in XXX (X) but not hypodermis (compare with N2 in Fig. 3). (E) *daf-12(rh273) dhIs64*, hypodermal *daf-9* is overexpressed in reproductively growing larva but (F) not expressed in Daf-c dauer larva. Worms are L3s and grown at 20°C, except where indicated.

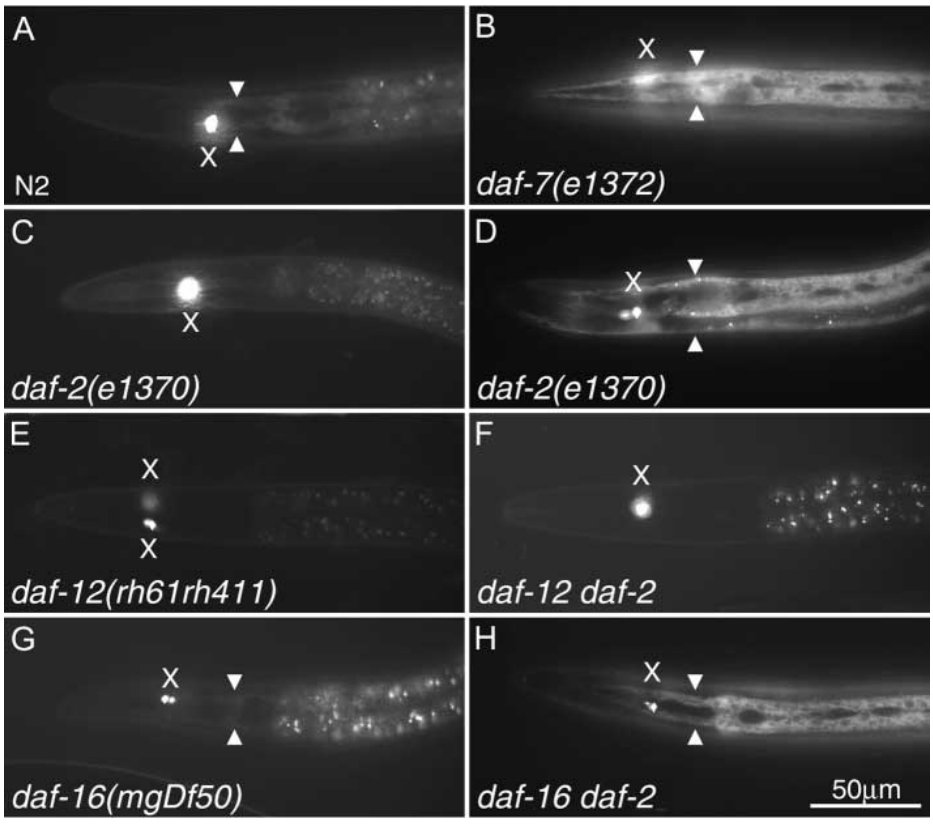


Fig. 6. Hypodermal *daf-9* regulation by genetic inputs. All panels are L3 reproductively growing larvae, except C, a dauer larva. XXX cells (X), hypodermis (arrowheads).

16/FOXO. For *daf-2* we used conditional temperature-sensitive Daf-c alleles, which are fully suppressed by the Daf-d null *daf-16(mgDf50)* (Gottlieb and Ruvkun, 1994; Ogg et al., 1997). Whereas hypodermal *daf-9* was strongly upregulated in *daf-2* reproductively growing animals relative to *daf-2(+)*, *daf-2* dauer larvae showed no hypodermal expression (Table 3, Fig. 6C,D). Alone, *daf-16(mgDf50)* had little effect on *daf-9* expression. Moreover, hypodermal upregulation was not *daf-16* dependent, as *daf-16daf-2* animals had elevated hypodermal expression at 20°C (Fig. 6G,H) and even higher expression at 25°C. By contrast, in *daf-2daf-12* animals, expression ceased (Fig. 6F) showing *daf-12* dependence.

TGFβ and cGMP signaling

Temperature sensitive Daf-c mutants of *daf-7*/TGFβ (Ren et al., 1996) and *daf-11*/guanylyl cyclase (Birnby et al., 2000) also upregulated hypodermal *daf-9* in reproductively growing animals at 20°C, but repressed expression in dauer larvae (Fig. 6B, Table 3). *osm-6*, a Daf-d locus that acts downstream of *daf-11* (Thomas et al., 1993) as well as *daf-3*/SMAD or *daf-5*/SNO, which mediate the transcriptional output of TGFβ signaling (da Graca et al., 2003; Patterson et al., 1997), had little effect on *daf-9* regulation. Moreover, *daf-5* only partly diminished hypodermal *daf-9* upregulation observed in the *daf-7* background (Table 3), while *daf-12* abolished expression. We conclude that reduced TGFβ signaling upregulates hypodermal *daf-9* primarily through *daf-12*. In addition, we noticed that the *dhIs64 daf-9::gfp* array enhanced the Daf-c phenotypes of *daf-2(e1370)* and *daf-7(e1372)* at normally semi-permissive temperatures, giving rise to 79±12% (n=504) and 79±18% (n=869) dauer larvae at 20°C, respectively, compared with 0% (n≥300) and 0% (n≥300) without the transgene. This may be because of the presence of multiple copies of the *daf-9* regulatory region, as hypodermal overexpression with heterologous promoters (see above) did not enhance Daf-c phenotypes. It could also reflect the effect of overexpression in XXX cells.

To address the possibility that TGFβ or insulin/IGF pathways substitute for one another, we crossed *dhIs64* into a *daf-3daf-16* double mutant (Table 3). Yet even in this background a normal response of hypodermal *daf-9* expression to temperature was seen. Thus, during reproductive growth, thermal influences on hypodermal expression can occur independently of major transcriptional outputs of TGFβ and insulin/IGF signaling.

Discussion

The hormone hypothesis posits that DAF-9/CYP450 produces a lipophilic hormone for the nuclear receptor DAF-12 (Gerisch et al., 2001; Jia et al., 2002). This hormone prevents diapause and fat deposition, and promotes gonadal maturation. In this work we show that *daf-9* exemplifies many features of an endocrine mechanism, notably, action at a distance, feedback regulation by its own signal, and dynamic regulation in response to environmental, physiological and genetic inputs. The molecular identities and properties of *daf-9* and *daf-12* provide some of the first functional evidence for lipophilic hormone signaling in the worm.

DAF-9 regulates diapause cell non-autonomously

Consistent with an endocrine mode of action, *daf-9* regulates diapause cell non-autonomously. Overexpressing *daf-9* constitutively in the hypodermis, using *col-3*, *dpy-7* and *wrt-1* promoters, efficiently rescued *daf-9* phenotypes. Animals bypassed diapause, displayed light intestines, normal gonadal development and near normal brood sizes. In mosaic experiments using *daf-9* constructs under control of the endogenous promoter, animals that retained hypodermal expression, but had lost expression in XXX cells reached reproductive maturity. Particularly revealing were C+ only mosaics, where *daf-9* expression was limited to hypodermis, body muscle and a few neurons. Despite the fact that most cells were genotypically *daf-9(–)*, tissues expressed reproductive programs. It is possible that our experiments overestimate the importance of the hypodermis simply because *daf-9* is overexpressed in transgenic arrays. Nevertheless, genotypic mosaics that contained *daf-9* on a cosmid at a fivefold lower gene dose also led to reproductive development when expressed in the C blastomere only. Thus, hypodermal expression of *daf-9* is sufficient to avert diapause and drive reproductive development, and *daf-9* acts cell non-autonomously.

daf-9 function in XXX cells also promotes reproductive development based on a number of lines of evidence. First, laser ablation of XXX cells leads to transient dauer-like arrest in a proportion of animals (Gerisch et al., 2001; Ohkura et al., 2003). However, the majority of such animals reach maturity, showing that XXX is important but not essential for diapause regulation. Such microsurgery experiments remove not only the *daf-9* signal, but others as well. To address this, we selectively expressed *daf-9* in XXX cells. Driven by an endogenous XXX regulatory element or heterologous *sdf-9* promoter, *daf-9* XXX variably rescued Daf-c and gonadal defects of *daf-9* mutants, confirming that expression in XXX cells promotes reproductive development. Again, because transgenes may overexpress *daf-9* it is unclear how closely this reflects the native situation.

In addition, the XXX cells also communicate with the hypodermis. Ablation of XXX cells (Gerisch et al., 2001) or mosaic loss of an extrachromosomal array upregulated *daf-9* hypodermal expression, showing that *daf-9* products from XXX cells normally inhibit hypodermal *daf-9*.

daf-9 works downstream of insulin/IGF and TGFβ signaling

Epistasis experiments reveal that *daf-9* loss of function acts downstream or parallel to *daf-16*/FOXO, *daf-3*/SMAD and *daf-5*/SNO, but upstream of *daf-12*, with respect to diapause (Gerisch et al., 2001; Jia et al., 2002). Overexpressing *daf-9* constitutively in the hypodermis confirmed and extended these observations. *daf-9* driven by *dpy-7* and *col-3* promoters efficiently rescued the Daf-c phenotypes of a null mutant in *daf-7*/TGFβ ligand, as well as in two hypomorphic mutants of the *daf-2*/insulin/IGF receptor. In the *daf-7* mutant and the less severe *daf-2* mutant, animals reached reproductive maturity. By contrast, overexpressing *daf-9* did not effectively suppress *daf-12* Mig phenotypes, consistent with *daf-9* action through *daf-12*.

Transcriptional regulation of hypodermal *daf-9* is likely to be a rate-limiting point of control in the hormone metabolic pathway in which this gene functions. That *daf-9* overexpression bypasses larval defects of insulin/IGF and TGFβ signaling mutants suggests that both pathways somehow act via activation of *daf-9*. Suppression of *daf-2* in particular suggests that analogous secondary endocrines in vertebrates might be able to ameliorate diabetic or other metabolic syndromes. Possibly PPARγ agonists work similarly to reverse cases of type 2 diabetes (Rocchi and Auwerx, 1999).

However, not all defects were suppressed by *daf-9* overexpression. For example, *daf-7* egg laying and dark intestine phenotypes prevailed. Although overt dauer morphogenesis was absent in rescued *daf-2(e1370)* strains, animals remained developmentally arrested as L3/L4 larvae with dark intestines typical of *daf-2* alone. Clearly both of these branches of the dauer pathway must also have *daf-9*-independent outputs.

Environmental signals regulate *daf-9*

Changes in *daf-9* expression are a striking visible readout for environmental influences on diapause, reflecting a central role in dauer regulation. As predicted, *daf-9* expression was sensitive to the environment, but primarily in the hypodermis. Here, by mid-L2 it was upregulated, signifying commitment to reproductive development, but switched off in the dauer larvae. Interestingly, the epidermis is a primitive endocrine tissue in many arthropods, including *Drosophila* (Lafont, 2000; Warren et al., 2002), and even in vertebrates steroidogenic enzymes are expressed in skin (Slominski et al., 1996).

Hypodermal *daf-9* expression is not, however, simply an on-off switch. Notably, under mild dauer-inducing conditions hypodermal *daf-9* was actually upregulated. One possibility is that this reflects a homeostatic response to ensure reproductive development in the face of mild adversity. Although the final choice between diapause and reproductive growth is not graded, but all or none (Antebi et al., 1998; Apfeld and Kenyon, 1998), animals must nevertheless adapt to different levels of stress. For example, preceding any final commitments to diapause, nematodes already shift to fat and carbohydrate

storage, lengthen the molt cycle and change their foraging behavior (Golden and Riddle, 1984; Thomas et al., 1993).

By contrast, *daf-9* expression in XXX cells increased in dauer larvae. This is surprising, as *daf-9* expression in XXX cells alone supported reproductive growth of *daf-9* mutants. Perhaps post-transcriptional regulation in XXX cells is key. Supporting this view, overexpression of *daf-9* in XXX cells alone, under the control of the endogenous and *sdf-9* promoters did not bypass the Daf-c phenotypes of *daf-2* and *daf-7*. Genetic analysis of the *sdf-9* phosphatase-like protein suggests that it post transcriptionally augments DAF-9 activity (Ohkura et al., 2003).

Dietary cholesterol influences dauer signaling

Aside from food, pheromone and temperature, evidently dietary cholesterol impacts hypodermal *daf-9* expression and dauer signaling. Cholesterol promotes reproductive development and fertility; deprivation arrests growth, impedes molting and decreases fertility (Shim et al., 2002; Yochem et al., 1999). In addition, reduced cholesterol phenocopies gonadal Mig and Daf-c defects in wild type, and enhances weak *daf-9* mutant phenotypes (Gerisch et al., 2001; Jia et al., 2002). Here, we found that although modest decreases in cholesterol also upregulated hypodermal *daf-9*, cholesterol starvation induced dauer formation and abolished *daf-9* expression.

Because cholesterol is the precursor to steroids, oxysterols, bile acids and vitamin D, cholesterol starvation is likely to perturb the production of sterol-derived hormones that regulate diapause. Interestingly, Niemann-Pick type C proteins mediate intracellular cholesterol trafficking (Ribeiro et al., 2001) and deletion of the two *C. elegans* homologs leads to a Daf-c phenotype (Sym et al., 2000). One of the homologues is reportedly expressed in the XXX cells (Ohkura et al., 2003). It is also possible that cholesterol availability indirectly influences the metabolism of a DAF-12 hormone.

DAF-12 regulates *daf-9* expression in a feedback loop

What is the molecular basis of *daf-9* hypodermal regulation? Notably, it was wholly dependent on *daf-12*, suggesting that DAF-12(+) positively promotes *daf-9* expression. By contrast, other Daf-d loci, such as *daf-16*, *daf-3* and *daf-5* alone, as well as *daf-16daf-3* double mutants had little effect. Moreover, DAF-12 may require some threshold level of a DAF-9-produced hormone to promote hypodermal expression, as such expression was absent in *daf-9* null mutants. *daf-12* dependence is somewhat surprising because by genetic epistasis it lies downstream of *daf-9*. Conceivably, within this endocrine tissue *daf-9* expression is DAF-12 regulated, but within downstream target tissues DAF-12 acts epistatically.

Other genetic evidence supports the view that DAF-12's activity on the *daf-9* promoter is regulated by negative feedback. First, hypomorphic *daf-9* alleles, which are predicted to diminish hormone production potently upregulated a *daf-9* promoter construct. Second, missense mutations in the *daf-12* ligand-binding domain, predicted to diminish affinity for ligand, also resulted in upregulation. If DAF-12 regulates the *daf-9* promoter directly, it suggests that DAF-12's transcriptional activity is inhibited by the *daf-9* hormone. Interestingly, an evolutionarily related vertebrate homolog, CAR-β is a constitutively active nuclear receptor the activity of which is inhibited by androstane ligands (Forman et al., 1998). Ecdysone receptor also initially positively promotes ecdysone production, but then negatively feeds back on synthesis (Lafont, 2000).

Daf-c mutants from insulin/IGF, TGFβ and cGMP pathways affected hypodermal *daf-9* expression much like environmental perturbations and *daf-9* mutants, causing upregulated hypodermal expression in reproductively growing animals but no expression in Daf-c dauer larvae. Notably, elevated *daf-9* expression was largely independent of the transcriptional outputs of these pathways, but dependent on *daf-12*. This observation suggests that insulin/IGF and TGFβ signaling cascades could directly regulate DAF-9 or DAF-12.

daf-9 has multiple larval functions

The role of *daf-9* in dauer formation was separable from its later role in gonadogenesis. For example, restoration of *daf-9* in XXX cells alone often led to dauer bypass, but subsequent gonadal defects. Additionally, cultures of *daf-9* mutants are typically either Daf-c or Mig, not mixtures of the two phenotypes. This could potentially be explained by the feedback regulation of *daf-9* described here. Although *daf-9* null mutants lack *daf-9* expression, hypomorphs may have increased hypodermal *daf-9*, as seen in *daf-9(rh50)*, thereby averting diapause. Potentially, lower levels of *daf-9* activity driven by the feedback loop are sufficient to bypass diapause, whereas higher levels are required to promote gonadal development. Alternatively, *daf-9* may be required at different

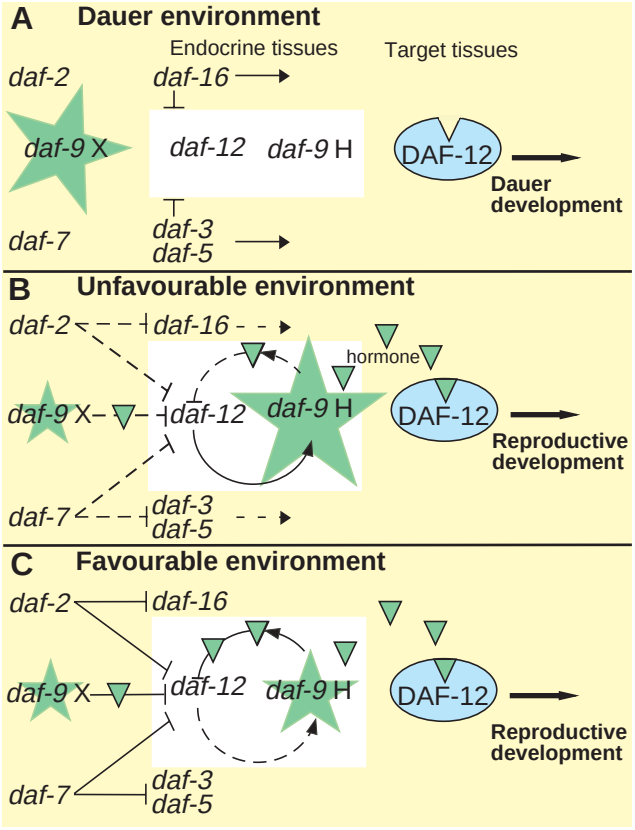


Fig. 7. Feedback model for diapause regulation (see Discussion). XXX cells (X) and hypodermis (H).

times, at the L3 commitment for the dauer decision, and L4 commitment for gonadal cell migration programs.

We interpret gonadal Mig phenotypes as a heterochronic delay in stage-specific migration programs, where cells repeat earlier steps and fail to advance to the next stage (Antebi et al., 1998). Such a program may regulate the general migratory machinery, as well as instructive surface receptors that guide cellular pathfinding in strict temporal sequence. Indeed, the UNC-5 receptor, repelled by ventral UNC-6/netrin cues, guides the distal tip cells dorsally, and is expressed in a delayed fashion or not at all in *daf-9* and *daf-12* ligand-binding domain mutants (Su et al., 2000). Evidently this program is advanced by hormonal signals. Interestingly, ecdysteroid signaling regulates the timing of border cell migration in the *Drosophila* ovary (Bai et al., 2000). Moreover, metastatic tumors can subvert hormonally activated migration programs (Green and Furr, 1999), and be influenced by UNC-5 status (Thiebault et al., 2003).

The dauer endocrine network

Our data suggest a three state model for regulation of diapause involving homeostatic feedback regulation of *daf-9* expression. In dauer-inducing conditions (Fig. 7A), environmental cues downregulate insulin/IGF, TGFβ and/or lipophilic hormone signaling below a crucial threshold. This leads to a failure to express hypodermal *daf-9*, possibly because DAF-12 activity is low or inactive. DAF-9 activity in XXX cells may also be similarly influenced. Consequently, reproductive hormone levels drop. In target tissues, unliganded DAF-12 specifies diapause. In conditions of moderate stress (Fig. 7B), initially low levels of reproductive hormone and/or TGFβ and insulin/IGF signaling result in compensatory upregulation of hypodermal *daf-9* by DAF-12. This drives reproductive programs, including the appropriate timing of gonadal distal tip cell migrations. In replete environments (Fig. 7C), *daf-9* reproductive hormone levels are high, and partly inhibit DAF-12 transcriptional effects on the *daf-9* promoter, keeping hormone levels within normal bounds. In reproductively growing larvae, XXX cells could secrete tonic levels of hormone, whereas hypodermis may respond dynamically to changes in XXX cell activity or directly to genetic and environmental inputs. It is possible that DAF-12 ligand-binding domain occupancy is a central integrator of this information. However, DAF-12 activity may also be modified directly by *daf-9*-independent inputs originating from TGFβ and insulin/IGF signaling.

The hormone hypothesis

Although a DAF-12 hormone has yet to be identified, the evidence in favor of one is compelling. First, global coordinated events mediated by *daf-9*/*daf-12* indicate endocrine control. Second, insulin/IGF and TGFβ receptors produce cell non-autonomous signals (Apfeld and Kenyon, 1998; Wolkow et al., 2000), and epistasis experiments placing *daf-9* and *daf-12* downstream suggest they play a role in this hormonal output (Gerisch et al., 2001; Jia et al., 2002). Third, lesions within the DAF-12 ligand-binding domain affecting predicted ligand contact residues imply that DAF-12 does indeed have a hormone (Antebi et al., 2000). Fourth, the molecular identities, epistasis and phenotypic overlap of *daf-9* and *daf-12* suggest a tight functional coupling (Antebi et al., 1998; Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002). Fifth, reduced cholesterol levels phenocopy *daf-9* mutant phenotypes in wild-type animals, consistent with production of a sterol hormone by *daf-9* (Gerisch et al., 2001). Sixth, expression of *daf-9* within limited cell types and *daf-12* throughout the body, imply that *daf-9* acts cell non-autonomously, proven here by mosaic and promoter studies. Finally, *daf-9* is autoregulated by negative feedback, a signature of endocrine systems. The next crucial step will be to isolate and biochemically identify the *daf-9* hormone.

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