Intercellular signaling of reproductive development by the *C. elegans* DAF-9 cytochrome P450

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Summary

Parallel pathways control *C. elegans* reproductive development in response to environmental cues. Attenuation of *daf-2* insulin-like or *daf-7* TGFβ-like signaling pathways cause developmental arrest at the stress resistant and long-lived dauer stage. Loss-of-function mutations in the cytochrome P450 gene *daf-9* also cause dauer arrest and defects in cell migration. A rescuing *daf-9*::GFP fusion gene driven by the *daf-9* promoter is expressed in two head cells at all stages, in the hypodermis from mid-second larval stage (L2) to the fourth larval stage (L4), and in the spermatheca of the adult hermaphrodite. Although the level of *daf-9*::GFP expression in the head cells and spermatheca is constant, hypodermal *daf-9*::GFP expression is modulated by multiple inputs. In particular, *daf-9*::GFP expression in the hypodermis is absolutely dependent on *daf-12*, the nuclear receptor that is negatively regulated by *daf-9* gene activity, suggesting feedback control between *daf-9* and *daf-12* in this tissue. *daf-9* expression exclusively in the hypodermis is sufficient to restore reproductive development in *daf-9* mutant animals, suggesting that *daf-9* functions in a cell nonautonomous manner. Furthermore, constitutive expression of *daf-9* in the hypodermis suppresses dauer arrest of *daf-7* mutant animals and inhibits dauer remodelling of some tissues in *daf-2* mutant animals. Thus, *daf-9* may integrate outputs from *daf-2* and *daf-7* signaling pathways to relay neuroendocrine signals through synthesis of a lipophilic hormone.

Key words: *daf-9*, *daf-12*, Dauer, Gonadal migration, Cytochrome P450, Insulin, *C. elegans*, TGFβ

Introduction

A wide range of metazoan signaling molecules relay information between neighboring cells or throughout the entire organism. One challenge is to understand how target cells interpret and integrate multiple incoming signals and respond accordingly. The nematode *C. elegans* employs multiple peptide and lipophilic hormones to coordinate differentiation of various tissues upon commitment to the reproductive or the developmentally arrested state. Under favorable growth conditions, *C. elegans* develops through four larval stages (L1-L4) to form a reproductive adult. However, unfavorable environmental conditions cause the animal to arrest at the dauer larval L3 stage (Riddle and Albert, 1997). The decision to develop via the alternative dauer stage requires the integration of multiple sensory inputs such as a small molecule dauer pheromone, food and temperature, and the execution of a remodelling program that affects the morphology and physiology of the animal. Molecular genetic analysis has identified an insulin-like pathway (Kimura et al., 1997; Pierce et al., 2001), a TGFβ-like pathway (Ren et al., 1996; Schackwitz et al., 1996) and a cGMP signaling pathway (Birnby et al., 2000), which are thought to couple sensory signals to the subsequent animal remodelling through transcriptional regulation of target genes. Downregulation of DAF-7 TGFβ ligand and DAF-2 insulin/IGF-I like receptor activity leads to activation of DAF-3 Smad protein and DAF-16 Forkhead transcription factor, respectively (Lin et al., 1997; Ogg et al., 1997; Patterson et al., 1997). In contrast to the detailed knowledge of the DAF-2 and DAF-7 signaling cascades, the mechanism by which these pathways are integrated remains largely unknown.

Genetic mosaic analysis showed that the DAF-2 insulin/IGF-I like receptor and the DAF-4 type II TGFβ receptor control reproductive development in a cell nonautonomous manner (Apfeld and Kenyon, 1998; Inoue and Thomas, 2000; Wolkow et al., 2000). A secondary signal is thought to be responsible for communication between the peptide hormone responsive tissues, such as the nervous system, and the rest of the body. Genetic analysis suggests that *daf-9* functions downstream of or in parallel to *daf-2* and *daf-7* and upstream of *daf-12* (Gerisch et al., 2001; Jia et al., 2002). *daf-9* encodes a cytochrome P450 enzyme, whereas *daf-12* encodes a nuclear receptor (Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002). As cytochrome P450 enzymes mediate steroid hormone synthesis in mammals and *Drosophila* (Miller, 1988; Warren et al., 2002), and because *daf-9* acts upstream of the nuclear receptor gene *daf-12* (Gerisch et al., 2001; Jia et al., 2002), DAF-9 may mediate the production of a lipophilic hormone that regulates DAF-12 activity. Based on its action downstream of *daf-2* and *daf-7* in the genetic epistasis analysis,

daf-9 expression or activity may in turn be regulated by the upstream *daf-2* and *daf-7* signaling pathways.

Two classes of *daf-9* mutant alleles have been described. Animals carrying strong loss-of-function alleles arrest as dauers unconditionally and seldom recover (Gerisch et al., 2001; Jia et al., 2002). Ultrastructural studies revealed that *daf-9* dauers display intermediate dauer morphology in selected tissues (Albert and Riddle, 1988). This suggests that parallel pathways may operate in conjunction with *daf-9* to complete the global remodelling in dauer animals. A second class of *daf-9* alleles confers weak loss-of-function phenotypes: reversible dauer arrest and a gonadal migration defect (Antebi et al., 1998; Gerisch et al., 2001; Jia et al., 2002). Allele and temperature specific extension of adult life span has also been reported for *daf-9* mutant animals (Gerisch et al., 2001; Jia et al., 2002). Furthermore, there are complex interactions between *daf-9*, germline and *daf-2* signaling pathways in the control of adult life span, reminiscent of those reported for *daf-12* (Larsen et al., 1995; Gems et al., 1998; Hsin and Kenyon, 1999).

The DAF-9 protein sequence is most similar to the mammalian CYP2 family of P450 enzymes, which are responsible for degradation of steroidal and xenobiotic compounds (Nebert and Russell, 2002). Nevertheless, DAF-9 is unlikely to be a functional homologue of the mammalian CYP2 enzymes. This is because *daf-9* expression is not induced by a range of xenobiotic compounds (Menzel et al., 2001), a feature of the mammalian CYP2 enzymes (Waxman, 1999). A biosynthetic role for DAF-9 was supported by the observations that cholesterol deprivation causes a gonadal migration defect, similar to that of hypomorphic *daf-9* mutant animals (Gerisch et al., 2001). Furthermore, cholesterol withdrawal inhibits recovery from dauer arrest by *daf-9* hypomorphs (Jia et al., 2002). This argues that *daf-9* may participate in the modification of cholesterol in the biosynthetic pathways to steroid hormones.

In this paper, we addressed the following questions. Where is the site of action of *daf-9* gene function? How does *daf-9* interact with the *daf-2* and *daf-7* signaling pathways? Is *daf-9* expression regulated at a transcriptional level? We addressed these questions by expressing a functional GFP-tagged DAF-9 protein under the control of the endogenous *daf-9* promoter and other well-established tissue-specific promoters. We find that *daf-9* directs larval development and gonadal migration in a cell nonautonomous manner and its action is intricately linked to *daf-2*, *daf-7* and *daf-12* activities.

Materials and methods

Strains

Strains used were as follows: wild-type N2 Bristol, *daf-7(e1372)* III, *daf-2(e1370)* III, *daf-1(m40)* IV, + / szT1 [*lon-2(e678)*] I; *daf-9(e1406) dpy-7(sc27)* / szT1 X, *dpy-7(sc27) daf-12(m20)* X, *daf-12(m20)* X, *daf-12(m583)* X.

mgEx661-662: *Ex[daf-9p::daf-9 genomic::GFP], mgEx663-664: Ex[dpy-7p::daf-9 cDNA::GFP; mec-7::GFP], mgEx669-670: Ex[sdf-9p::daf-9 cDNA::GFP*; *mec-7::GFP], mgEx665-666*: *Ex[che-2p::daf-9 cDNA::GFP; mec-7::GFP]* and *mgEx667-668: Ex[col-12p::daf-9 cDNA::GFP; mec 7::GFP]*.

In all tables, line 1 refers to the odd-numbered extrachromosomal array and line 2 refers to the even-numbered extrachromosomal array.

Generation of *daf-9p*::*daf-9*::GFP transgenic lines

The genomic region spanning all exons of *daf-9* plus 7 kb of non-coding sequence 5′ to the initiator codon of the *daf-9*b isoform was amplified by PCR and subcloned into the *Sal*I/*Bgl*II sites of pPD95.69 (kindly provided by A. Fire) in two steps. The nuclear localisation signal of pPD95.69 was removed as a result. The genomic region spanning the first exon, the first intron and seven residues of the second exon together with 3 kb of non-coding sequence 5′ to the initiator codon of the *daf-9*b isoform was amplified by PCR and subcloned into the *Bgl*II site of pPD95.75 (kindly provided by A. Fire). The ligation junctions of the above constructs were sequenced to ensure that the *daf-9*-coding sequence was in-frame with the GFP coding sequence. The genomic region encompassing all exons of the *daf-9* plus 3 kb of non-coding sequence 5′ to the initiator codon of *daf-9*b isoform was amplified by PCR and subcloned into the *Bgl*II/*Age*I sites of pPD95.75. Intron 1 of the *daf-9*b isoform was removed from the last construct by recombinant PCR and the product subcloned into the *Bgl*II/*Age*I sites of pPD95.75. The nuclear receptor consensus half site in intron 1 of the *daf-9*b isoform was mutated into a LexA binding site by recombinant PCR and the product subcloned into the *Bgl*II/*Age*I sites of pPD95.75. For the last three constructs, all exons and introns of *daf-9* were fully sequenced.

The above constructs were injected into N2 wild-type animals at 10 to 30 ng/μl. pBluescript was used to normalise the total concentration of injection mix to 100 ng/μl. Extrachromosomal arrays which gave robust *daf-9*::GFP expression were introduced into + / szT1 [*lon-2(e678)*]; *daf-9(e1406) dpy-7(sc27)* / szT1 animals by genetic crosses.

Generation of tissue specific *daf-9*::GFP transgenic lines

Tissue specific *daf-9*::GFP transgenic constructs were generated by assembling three PCR products by a modified recombinant PCR method (Hobert, 2002). The following tissue specific promoters were amplified from N2 genomic DNA: *sdf-9* (3.7 kb), *che-2* (2.5 kb), *dpy-7* (0.4 kb) and *col-12* (1 kb) (Johnstone and Barry, 1996; Fujiwara et al., 1999; Ohkura et al., 2003). The primer sequences defining the 5′ end of the promoter in these transgenes are as follows:

sdf-9, 5′-tcaaaaatacattatggcgactc-3′;
che-2, 5′-gtcacacatgaatgagtctcgcc-3′;
dpy-7, 5′-tcattccacgatttctcgcaac-3′; and
col-12, 5′-gaaagttcagaactggcatggag-3′.

Each promoter fragment encompasses sequence immediately 5′ to the start codon of the respective gene. GFP-coding sequence and *unc-54* 3′ UTR sequence were amplified by PCR using pPD95.75 as template. The coding sequence of the *daf-9b* isoform was amplified by PCR using a *daf-9* cDNA clone as template (kindly provided by Y. Kohara). Purified PCR products were injected into N2 wild-type animals at 5 ng/μl in the presence of *mec-7*::GFP plasmid at 30 ng/μl. pBluescript was used to normalise the total concentration of injection mix to 100 ng/μl. Extrachromosomal arrays which gave robust *daf-9*::GFP expression were introduced into + / szT1 [*lon-2(e678)*]; *daf-9(e1406) dpy-7(sc27)* / szT1 animals by genetic crosses.

Assay for dauer arrest

Adults were allowed to lay eggs on nematode-growth plates for 3 hours at room temperature, and progeny were incubated at 20°C for 68 and 92 hours or 25°C for 55 hours. Dauers were distinguished by a radially constricted body, dauer alae and a constricted pharynx. Dauer assays for each strain were repeated at least three times.

Lifespan assay

Adult lifespan of various strains was determined at 25°C, with agar plates containing 0.1 g/ml FUDR to prevent growth of progeny. Synchronous populations of worms from 3 hour egglays on nematode-growth medium plates were allowed to develop at 15°C until young adult stage, before being transferred to FUDR plates and shifted to 25°C. Worms were monitored every 2-4 days and were scored as dead when they no longer responded to gentle prodding with a platinum wire. Lifespan is defined as the time elapsed from the day when

worms were put on FUDR plates (day 0) to when they were scored as dead. Worms that crawled off the plates were excluded from calculations. Lifespan assays were repeated at least twice.

Results

daf-9 controls dauer arrest cell-nonautonomously

We generated a *daf-9*::GFP fusion gene that bears the *daf-9* 5′ regulatory region, the entire *daf-9*-coding sequence, including introns, and fuses GFP to the last amino acid residue of the DAF-9 protein. This translational fusion gene rescues the strong loss-of-function *daf-9(e1406)* mutation (Table 1), suggesting that visualization of the fluorescent fusion protein should reveal where the functional gene product acts. *daf-9(e1406)* animals carrying the fusion gene developed into reproductive adults, in contrast to their non-transgenic siblings that arrest as dauer larvae irreversibly. Consistent with previous reports, *daf-9*::GFP is expressed in a pair of cells in the anterior ganglion in L1 larvae, which persists in all larval stages and in adults, in the hypodermis from the mid-L2 stage to the end of L4 stage, and in the spermatheca of adult hermaphrodites (Gerisch et al., 2001; Jia et al., 2002). The *daf-9*::GFP-expressing head cells have been identified as XXXL/R, which are thought to be embryonic hypodermal cells (Ohkura et al., 2003). Nevertheless, we favour the assignment of XXXL/R cells as neuron-like because they possess axon-like projections, and *daf-9*::GFP in these cells are refractory to feeding RNAi against *daf-9* in larvae and adult animals (data not shown), two characteristics of neuronal cells (Fraser et al., 2000; Tavernarakis et al., 2000; Timmons et al., 2001).

daf-9 encodes a cytochrome P450 enzyme and is proposed to synthesize a lipophilic hormone. Given its spatially restricted expression pattern, DAF-9 may fulfill its role in preventing dauer arrest by mediating the maturation or destruction of a hormonal signal that acts in a cell-nonautonomous manner. To address this, we generated a series of promoter fusion genes where *daf-9* cDNA expression was driven by tissue-specific heterologous promoters, and tested whether they rescue the dauer constitutive phenotype of *daf-9(e1406)* mutant animals.

We used the collagen gene *dpy-7* promoter to direct hypodermal expression of *daf-9* during larval stages (Gilleard et al., 1997). A GFP tag was appended to the DAF-9 C terminus so that tissue specific DAF-9 expression could be confirmed by visualization of the GFP signal. Restoration of *daf-9* activity in the hypodermis alone was sufficient to prevent dauer arrest of *daf-9(e1406)* mutant animals (Table 1); these transgenic animals developed into reproductive adults with the same growth rate as animals carrying a *daf-9* transgene driven by its own promoter. Next, we expressed *daf-9* exclusively in XXXL/R cells under the control of the *sdf-9* gene promoter (Ohkura et al., 2003). At 20°C, *daf-9* expression in the two XXXL/R cells was sufficient to prevent dauer arrest of *daf-9(e1406)* mutant animals (Table 1). Nevertheless, 5-20% of *daf-9(e1406)* animals that expressed *daf-9* in the XXXL/R cells arrested as dauers at 25°C (*mgEx667*, *mgEx668*; *n*=898). This was not due to downregulation of the *sdf-9p*::*daf-9*::GFP transgene at 25°C as no dramatic diminution of GFP fluorescence was observed in the XXXL/R cells. By contrast, *dpy-7* promoter-driven, *daf-9* expression in the hypodermis led to complete suppression of dauer arrest of *daf-9(e1406)* animals at 25°C (*mgEx663, mgEx664*; *n*=300). Therefore, hypodermal *daf-9* expression or a general increase in *daf-9* expression level may be crucial in promoting reproductive development at elevated, dauer-inducing temperatures. Taken together, *daf-9* activity in the hypodermis is sufficient to orchestrate reproductive development in an otherwise *daf-9* deficient animal. Hence, our observations support the hypothesis that *daf-9* inhibits dauer arrest in a cell nonautonomous manner.

daf-9 controls gonadal migration cell-nonautonomously

In contrast to the strong loss-of-function allele *daf-9(e1406)*, *daf-9* weak loss of function alleles confer a gonadal migration defect, where the distal tip cells fail to migrate dorsally at the third larval stage (Gerisch et al., 2001; Jia et al., 2002). This indicates that *daf-9* also plays a role in gonadal migration during reproductive development. Transgenic expression of *daf-9* under the control of its endogenous promoter fully rescued *daf-9(e1406)* mutant animals and no gonadal migration defect was observed at the L4 or adult stage (Table 2). Similarly, normal gonadal migration was observed in *daf-9(e1406)* mutant animals when *daf-9* was expressed exclusively in the hypodermis or the XXXL/R cells under the control of *dpy-7* or *sdf-9* promoters, respectively (Fig. 1A and Table 2). Taken together, our results suggest that *daf-9* acts in a cell nonautonomous manner to direct the movement of the distal tip cells that drive proper gonadal migration, and that expression

Table 1. Rescue of *daf-9(e1406)* with extrachromosomal arrays (20°C 68 hours post egg lay)

	% transgenic non-dauers (*n*)*	
Transgene	Line 1	Line 2
–†	0 (>200)	–
*daf-9*p::*daf-9 genomic*::GFP	100 (264)	100 (416)
*dpy-7*p::*daf-9* cDNA::GFP	100 (412)	100 (185)
*sdf-9*p::*daf-9* cDNA::GFP	97 (320)	98 (557)
*che-2*p::*daf-9* cDNA::GFP‡,§	62 (145)	44 (323)

*The genetic background was *daf-9(e1406) dpy-7(sc27)*.

†In addition to no transgene control, two control *Ex[mec-7::GFP]* transgenes did not rescue *daf-9(e1406)*. No Dpy non-dauer progeny from *daf-9(e1406) dpy-7(sc27)/szT1 Ex[mec-7p::GFP]* was observed (total number of brood examined=37).

‡All other transgenic animals not scored as non-dauers were partial dauers.

§% transgenic non-dauers at 92 hours post egg lay are: line 1, 79%; line 2, 84%.

Table 2. Gonadal migration phenotype (20°C 92 hours post egg lay)

	% normal gonadal migration (*n*)*,†	
Transgene	Line 1	Line 2
–	0 (>200)	–
*daf-9*p::*daf-9 genomic*::GFP	100 (>200)	100 (>200)
*dpy-7*p::*daf-9* cDNA::GFP	100 (412)	100 (185)
*sdf-9*p::*daf-9* cDNA::GFP	97 (311)	100 (544)
*che-2*p::*daf-9* cDNA::GFP	34 (110)	12 (292)

*The genetic background was *daf-9(e1406) dpy-7(sc27)*.

†Normal gonadal migration is scored when both gonad arms display proper ventral to dorsal migration.

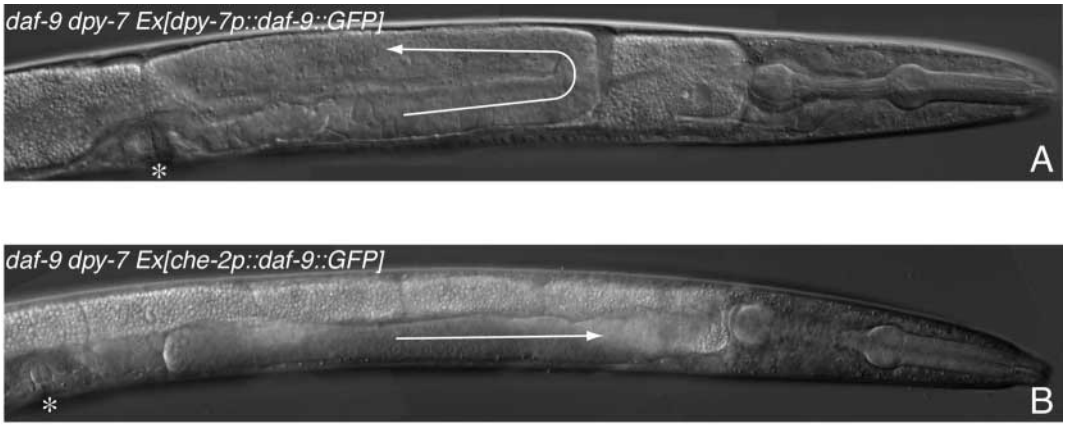

Fig. 1. Rescue of *daf-9* gonadal migration phenotype. (A) Hypodermal expression of *daf-9*, directed by a *dpy-7*p::*daf-9*::GFP transgene, was sufficient to rescue the gonadal migration phenotype of *daf-9(e1406) dpy-7(sc27)* mutant animals. Shown is a late L4 larva as indicated by the vulval morphology (marked with an asterisk). (B) Expression of *daf-9* in a subset of sensory neurons, directed by a *che-2*p::*daf-9*::GFP transgene, failed to rescue the gonadal migration phenotype of *daf-9(e1406) dpy-7(sc27)* mutant animals. Shown is a late L4 larva, as indicated by the vulval morphology (marked with an asterisk).

either from the broadly distributed hypodermal cells or the anterior XXXL/R cells supplies sufficient signal to do so.

daf-9 expression in ciliated sensory neurons partially rescues *daf-9* mutant animals

To test whether DAF-9 expression can confer endocrine function to other tissues, we expressed *daf-9* in 56 ciliated neurons using the *che-2* gene promoter that is inactive in XXXL/R cells (Fujiwara et al., 1999; Ohkura et al., 2003). Ectopic *daf-9* activity in the ciliated sensory neurons was sufficient to prevent dauer arrest of the majority of *daf-9(e1406)* mutant animals (Table 1). Only a small fraction of transgenic animals arrested as partial dauers. This suggests that DAF-9 can indeed function in ciliated sensory neurons and allow production of the putative lipophilic hormone that promotes reproductive development.

Even though expression of *daf-9* in the ciliated neurons using the *che-2* promoter rescued *daf-9(e1406)* dauer arrest, more than 70% of the reproductive adults displayed a gonadal migration defect (Fig. 1B and Table 2). Such defect is also observed in animals bearing *daf-9* weak loss-of-function alleles. One possibility is that the *daf-9*-expressing cells and the target distal tip cells are too distant from each other. Notably, of the 56 neurons where the *che-2* promoter is active, 49 of them are located in the head, five in the tail and none in the vicinity of the distal tip cells (Fujiwara et al., 1999). However, this is ruled out by the complete suppression of gonadal migration phenotype of *daf-9* mutant animals when *daf-9* activity was restored only in the two XXXL/R cells in the head (Table 2). We therefore attribute the gonadal migration defect to a suboptimal level of lipophilic hormone that is produced by DAF-9 in ciliated neurons. Alternatively, the complement of enzymes in ciliated sensory neurons may only be able to synthesize a hormone that promote non-dauer fate but not proper gonadal migration, unlike the ones in the native XXXL/R cells or hypodermis.

Effect of constitutive *daf-9* expression on dauer arrest in *daf-7*(–) and *daf-2*(–) mutant animals

Genetic analysis suggests that *daf-9* functions either downstream of or in parallel to *daf-16* and *daf-3* in the dauer pathway (Gerisch et al., 2001; Jia et al., 2002). One attractive model is that *daf-9* expression is regulated at a transcriptional level by DAF-16 and DAF-3 in response to the *daf-2* insulin-like and *daf-7* TGFβ like signaling pathways, respectively. Modulation of *daf-9* gene expression would in turn alter the level of a probable lipophilic secondary hormonal signal that promotes reproductive development. If this is the case, constitutive expression of DAF-9 may substitute for a loss of *daf-7* or *daf-2* signaling and direct reproductive development in *daf-7*(–) and *daf-2*(–) mutant animals, which normally arrest as dauers.

To uncouple *daf-9* expression from any potential transcriptional control originating from the *daf-7* and *daf-2* pathways, we introduced transgenes that direct *daf-9* expression in the hypodermis or the XXXL/R cells under the control of *dpy-7* or *sdf-9* promoters, respectively, into *daf-7(e1372)*, *daf-1(m40)* and *daf-2(e1370)* mutant animals. The same transgenes were fully functional in rescuing the dauer phenotype and gonadal migration defect of *daf-9(e1406)* mutant animals.

The *daf-7* and *daf-1* genes encode a TGFβ like ligand and a type I TGFβ receptor, respectively (Georgi et al., 1990; Ren et al., 1996). Constitutive hypodermal expression of *daf-9* suppressed the dauer arrest phenotype of *daf-7(e1372)* mutant animals (Table 3). At 25°C, none of the transgenic animals arrested at the dauer stage, and the majority of them became gravid adults while all non-transgenic *daf-7(e1372)* mutant animals arrested as dauers. Similar results were obtained when the same transgenes were introduced into *daf-1(m40)* animals. By contrast, *daf-9* expression in the XXXL/R cells, verified by GFP fluorescence of the DAF-9::GFP fusion protein, was unable to prevent dauer arrest of *daf-7(e1372)* mutant animals at 25°C (Table 3), although a fraction of the dauers did recover spontaneously upon prolonged incubation. Our results demonstrate that constitutive *daf-9* hypodermal, but not XXXL/R, expression can substitute for the loss of *daf-7* neuroendocrine signal and bypass the block in TGFβ signaling in target tissues. This argues a major role for *daf-9* in

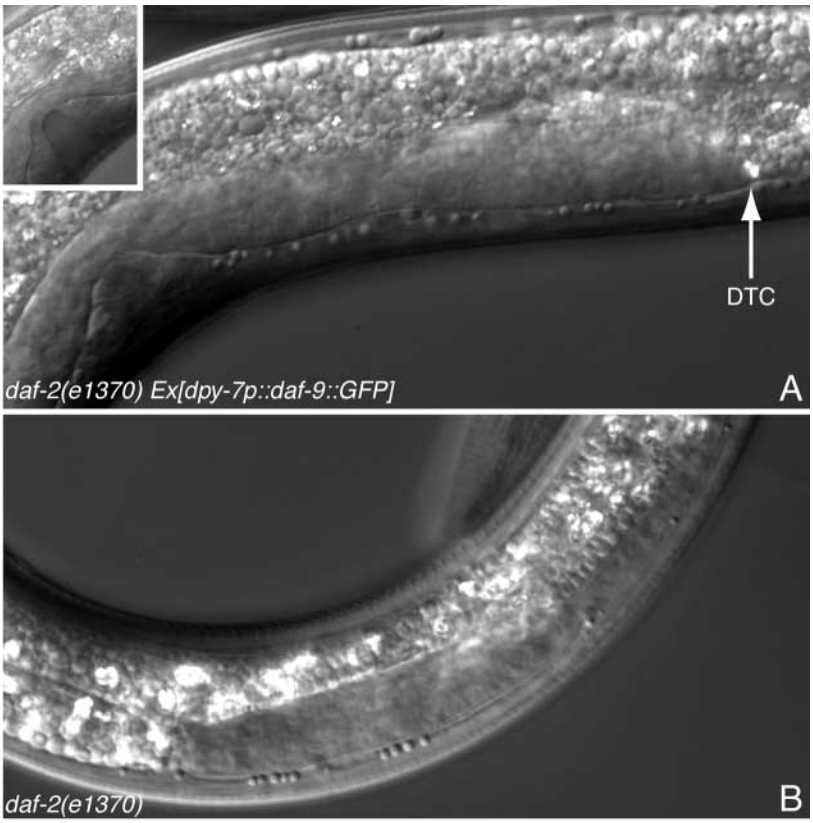

Fig. 2. Partial suppression of *daf-2* dauer arrest by *daf-9* expression in the hypodermis. (A) A *daf-2(e1370)* partial dauer animal carrying a *dpy-7*p::*daf-9*::GFP transgene. Although the vulva displayed L4 morphology (inset), gonadal migration was arrested and abundant refractile bodies, indicative of lipid accumulation, were apparent in the intestinal cells. The arrow indicates the position of the distal tip cell (DTC). (B) A *daf-2(e1370)* dauer animal showing arrested vulval development, gonadal migration and refractile bodies in the intestinal cells. All animals were raised at 25°C.

transducing the *daf-7* signal, perhaps through production of a lipophilic hormone in the hypodermis. Alternatively, *daf-9* may act in parallel of *daf-7* in promoting reproductive development.

daf-2(e1370) animals carry a missense mutation in the kinase domain of the *C. elegans* insulin receptor homologue (Kimura et al., 1997). All *daf-2(e1370)* animals arrest at the dauer stage when grown at 25°C. Expression of *daf-9* exclusively in the XXXL/R cells was unable to suppress the dauer arrest phenotype (n=278). However, constitutive expression of *daf-9* in the hypodermis, under the control of the *dpy-7* promoter, partially suppressed the dauer arrest phenotype of *daf-2(e1370)* mutant animals (Fig. 2). Although no transgenic animals became reproductive adults, they displayed non-dauer characteristics in a tissue-specific manner. At 25°C, *daf-2(e1370)* animals complete molting into dauers at ~52 hours post egg laying. At this time, sporadic pharyngeal pumping persisted in transgenic animals expressing DAF-9 in the hypodermis, indicating that they were not completely transformed into arrested dauers. After 90 hours of development, individual tissues of these transgenic animals and their non-transgenic siblings were examined under high magnification using Nomarski optics. Non-transgenic *daf-2(e1370)* dauer had a constricted pharynx and a highly refractile intestine because of accumulation of lipids. Moreover, vulval and germline development were arrested and dauer alae were visible on the cuticle. By contrast, *daf-2(e1370)* transgenic animals overexpressing DAF-9 in the hypodermis, the pharynx was not constricted and no dauer alae were observed. However, the intestine was highly refractile and resembled that of a dauer animal. It was striking to note that although the vulva of some of the transgenic animals displayed L4 morphology (36%, n=76), germline development was arrested and the distal tip cells failed to reflex. Taken together, these data show that *daf-9* expression in the hypodermis could compensate for the loss of *daf-2* activity and promote reproductive development of the hypodermis, pharynx and vulva. This implies that *daf-9* may act downstream of or in parallel to the *daf-2* signaling pathway and specify non-dauer fate in a subset of tissues. However, the lipophilic hormone processed by DAF-9 in the hypodermis is unlikely to overcome the lack of *daf-2* signaling in the intestine and germline. Our results are consistent with mosaic analysis which suggests a cell-autonomous role of *daf-2* in the reproductive development of P_1-derived germline and gonad (Apfeld and Kenyon, 1998).

Table 3. Effect of *daf-9* overexpression on dauer formation (25°C 55 hours post egg lay)

	% transgenic non-dauers (n)	
	Line 1	**Line 2**
***dpy-7*p::*daf-9* cDNA::GFP**		
daf-7(e1372)†	100 (140)	74 (210)*
daf-1(m40)†	100 (124)	100 (122)
***sdf-9*p::*daf-9* cDNA::GFP**		
daf-7(e1372)†	0 (101)	0.4 (488)

*All other transgenic animals not scored as non-dauers were partial dauers.
†All non-transgenic animals were dauers.

Effect of constitutive *daf-9* expression on aging in *daf-2*(–) animals

In addition to the dauer arrest phenotype, *daf-2(e1370)* animals have a life span twice as long as wild-type animals (Kenyon et al., 1993; Larsen et al., 1995). We tested the hypothesis that *daf-9* may act in the *daf-2* signaling pathway to control adult life span. Expression of *daf-9* in the hypodermis was driven by *col-12* or *dpy-7* promoters in *daf-2(e1370)* animals. The *col-12* promoter is active in larval stages and in the first 2 days of adulthood, whereas the *dpy-7* promoter is active only in larval stages (Johnstone and Barry, 1996). Transgenic and non-transgenic *daf-2(e1370)* animals were allowed to develop at the permissive temperature (15°C) until the late L4 stage, and then

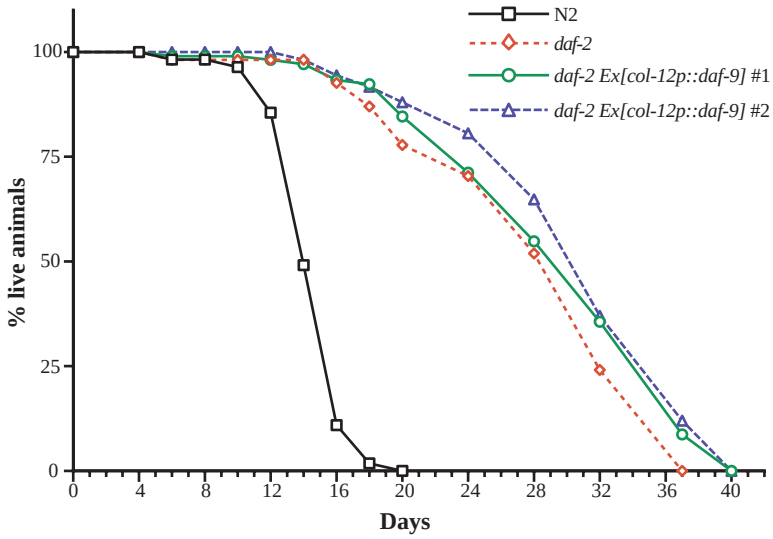

Fig. 3. Effect of *daf-9* overexpression on life span of *daf-2* animals. Adult lifespan of N2 (*n*=55), *daf-2(e1370)* (*n*=54) and *daf-2(e1370)* Ex[*col-12*p::*daf-9*::GFP] (#1, *n*=104; #2, *n*=108) were determined at 25°C. Two independent trials were conducted and the result of one trial is shown.

shifted to the restrictive temperature (25°C) for determination of adult life span. In four independent trials, *daf-2(e1370)* animals expressing *daf-9* in the hypodermis from the *col-12* or *dpy-7* promoters (*n*=351) had a similar adult life span as their non-transgenic siblings (*n*=204) (Fig. 3 and data not shown). In conclusion, we found no evidence that *daf-9* acts downstream of *daf-2* in the control of adult life span.

Transcriptional control of *daf-9* gene expression

We monitored *daf-9* expression in XXXL/R cells, hypodermis and spermatheca, using a functional *daf-9*::GFP fusion gene that contains ~7 kb of the endogenous *daf-9* promoter as well as all exons and introns of *daf-9* (construct i, Fig. 4E). This transgene rescued the strong loss of function mutation *daf-9(e1406)*, suggesting that it contains all the sequence elements for proper expression of *daf-9*. When this transgene was introduced into *daf-2(e1370)* and *daf-7(e1372)* mutant animals grown at the non-permissive temperature (25°C), we found that hypodermal expression of *daf-9*::GFP was absent in dauers, even though the *daf-9*::GFP expression in XXXL/R persisted. The lack of hypodermal *daf-9*::GFP expression was also noted in wild-type dauer animals derived from starvation. The *daf-9*::GFP transgene was unable to suppress dauer arrest of *daf-2(e1370)* and *daf-7(e1372)* animals (*n*>100) at the restrictive temperature (25°C) and enhanced dauer arrest of these animals at the permissive temperature (15°C). At 15°C, *daf-2(e1370)* and *daf-7(e1372)* transgenic animals displaying hypodermal *daf-9*::GFP expression entered the reproductive program without delay, whereas animals lacking such expression arrested as dauer for prolonged periods (>7 days). We speculate that *daf-2* and *daf-7* signaling pathways may play a role in controlling the hypodermal expression of *daf-9*, which is crucial in the commitment of reproductive development. In *daf-2(e1370)* and *daf-7(e1372)* animals, *daf-9* expression driven by its endogenous promoter may be compromised and therefore insufficient to initiate the reproductive program.

daf-9 was placed upstream of *daf-12* by genetic epistasis analysis (Gerisch et al., 2001; Jia et al., 2002). As feedback regulation of cytochrome P450 genes by nuclear receptor is well documented (Waxman, 1999; Chawla et al., 2001), we wondered if *daf-9* could be under the transcriptional control of DAF-12 nuclear receptor. We monitored *daf-9* expression using a functional *daf-9*::GFP fusion gene (construct i, Fig. 4E). When this transgene was introduced into animals carrying the strong loss of function allele *daf-12(m20)* (Antebi et al., 1998), a dramatic reduction of hypodermal expression of *daf-9*::GFP was observed at 15°C and 25°C, while expression in the XXXL/R cells and spermatheca persisted (Fig. 4A-D, Table 4, and data not shown). Similar results were obtained in *daf-12(m583)* mutant animals (data not shown). Hence, DAF-12 activates *daf-9* gene expression in the hypodermis. Furthermore, *daf-9* and *daf-12* appear to form a feedback regulatory loop in which DAF-12 is downregulated by an increase in antagonistic ligand production by DAF-9.

What is the response element in the *daf-9* promoter at which DAF-12 exerts its transcriptional control? As DAF-12 positively regulates *daf-9* expression in the hypodermis, deletion of such an element should result in diminution of *daf-9* hypodermal expression in wild-type animals. To this end, a series of transcriptional and translational *daf-9*::GFP transgenes were constructed (Fig. 4E). The spatial and temporal expression pattern of *daf-9* could be fully recapitulated by a transgene containing 3 kb of promoter sequence plus intron 1 of the b isoform of *daf-9*. However, deletion of intron 1 resulted in a dramatic reduction of hypodermal *daf-9*::GFP expression, reminiscent of that observed in *daf-12*(–) animals. We were unable to quantitate the signal as the GFP fluorescence was readily bleached upon excitation. Interestingly, the removal of intron 1 also led to the elimination of *daf-9*::GFP expression in the spermatheca. We noticed a nuclear receptor consensus half site (TGTTCT) within intron 1 that is also evident in an analogous location in the *C. briggsae daf-9* gene. To test whether this sequence represents a DAF-12-binding site, we mutated it from GTGTTCTGT into a LexA-binding site (GTACTGTAT) in the context of a rescuing *daf-9*::GFP fusion gene that is normally expressed in XXXL/R, hypodermis and spermatheca (Fig. 4E, construct v). Surprisingly, elimination of the nuclear receptor half site led to a loss of *daf-9*::GFP expression in the spermatheca alone (three extrachromosomal arrays, 25°C, hypodermal

Table 4. Hypodermal expression of *daf-9*::GFP (L2 larvae)

	% animals expressing hypodermal *daf-9*::GFP (*n*)	
*daf-9*p::*daf-9* genomic::GFP	15°C	25°C
Wild type	93 (136)	96 (80)
dpy-7(sc27) daf-12(m20)	19 (247)	5 (127)

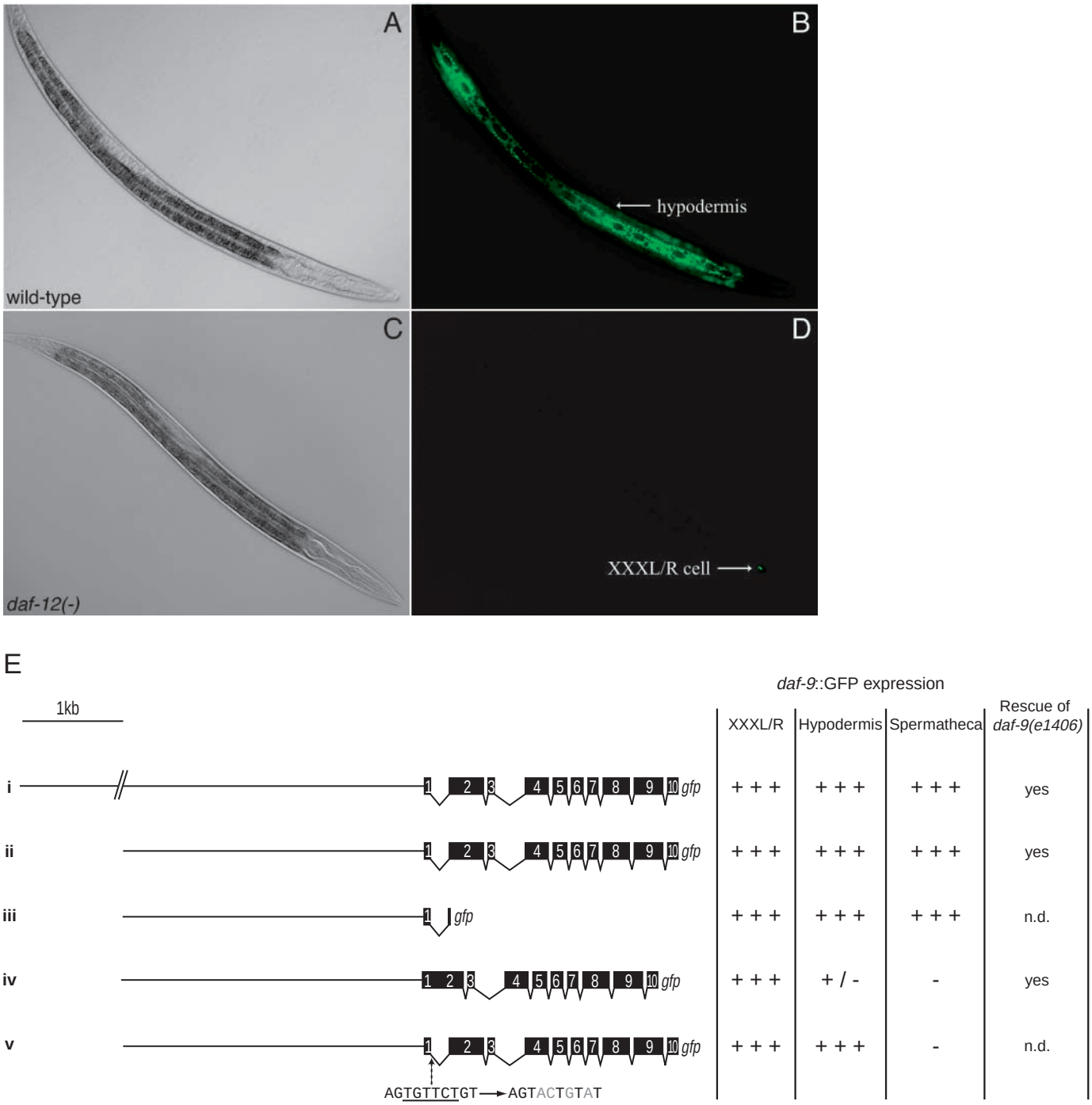

Fig. 4. Transcriptional control of *daf-9* by *daf-12*. (A,B) An N2 wild-type larva carrying a *daf-9*p::*daf-9 genomic*::GFP transgene at late L2 stage. (A) Normaski image. (B) Fluorescence image showing strong *daf-9*::GFP expression in the hypodermis. (C,D) A *dpy-7(sc27) daf-12(m20)* larva carrying a *daf-9*p::*daf-9 genomic*::GFP transgene at late L2 stage. (C) Normaski image. (D) Fluorescence image showing *daf-9*::GFP expression in the XXXL/R cell but not in the hypodermis. (E) Summary of *daf-9*::GFP expression pattern driven by five transgenes that encompass different promoter, exonic and intronic sequence from the endogenous *daf-9* locus. In construct v, a nuclear receptor (NR) consensus half site (underlined) in intron 1 was mutated to a LexA-binding site with the altered bases in grey. Transgenes i, ii and iv rescued the dauer arrest phenotype of *daf-9(e1406) dpy-7(sc27)* mutant animals. n.d., not determined.

expression=90% *n*=375; spermathecal expression=2% *n*=155). This suggests that the sequence TGTTCT is critical for spermathecal *daf-9* expression. However, *daf-9* hypodermal expression is unlikely to be controlled by DAF-12 through binding to the same sequence. Taken together, the 181bp of intron 1 sequence plays a major role in directing *daf-9* expression in the hypodermis and spermatheca. Although *daf-12*, in part, appears to control the hypodermal

expression, an unknown factor is likely to bind to a TGTTCT element in *daf-9* intron 1 and govern the spermathecal expression.

Discussion

Genetic analysis shows that *daf-9* acts downstream of or in parallel to *daf-2* insulin-like and *daf-7* TGFβ like signaling pathways to control a developmental decision between reproductive development versus arrest at the dauer diapause stage, as well as metabolism and life span in *C. elegans* (Gerisch et al., 2001; Jia et al., 2002). Here, we have demonstrated that *daf-9* regulates dauer arrest and gonadal migration in a cell nonautonomous manner. This was achieved by overexpressing a DAF-9::GFP fusion protein in specific tissues of genetically *daf-9*(–) animals. Our results support the hypothesis that DAF-9 cytochrome P450 enzyme acts in the synthesis pathway of a lipophilic ligand. Such a lipophilic ligand may well serve as a secondary signal which mediates, at least in part, *daf-2* and *daf-7* signaling pathways as overexpression of *daf-9* suppresses the dauer arrest phenotype of *daf-7*(–) animals and allows reproductive development of a subset of tissues in *daf-2*(–) animals. Finally, we provided evidence that DAF-12 nuclear receptor is engaged in feedback regulation with DAF-9 cytochrome P450 through transcriptional activation of *daf-9* expression in the hypodermis.

daf-9 acts upstream of *daf-12* and antagonizes its activity in the dauer pathway (Gerisch et al., 2001; Jia et al., 2002). Two simple models consistent with the genetic analysis are that *daf-9* participates in the synthesis of a *daf-12* antagonist, or that *daf-9* degrades a *daf-12* agonist. We favour the former model. First, expression of *daf-9* in single tissues is sufficient to direct reproductive development of other tissues, suggesting that *daf-9* mediates the synthesis of an endocrine signal. Second, *daf-9* expression is restricted to three tissues, while a ubiquitous expression pattern was reported for *daf-12* (Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002). It is conceivable that once the *daf-12* ligand is generated in XXXL/R, hypodermis or spermatheca by DAF-9, it would diffuse to target tissues via the pseudocoelom. At this stage, we cannot exclude the possibility that the DAF-12 ligand may be degraded by DAF-9. However, this necessitates transport of the DAF-12 ligand throughout the body to the three *daf-9*-expressing tissues for destruction. Another model involves co-expression of *daf-9*, and other P450 enzyme(s) that are genuinely involved in *daf-12* ligand synthesis, in the same tissue. The level of agonist produced, and DAF-12 transcriptional potential, would then depend on the relative activity of the competing P450 enzymes. This is similar to cases in *Drosophila* and mammals, where hormone availability is modulated locally by enzymes, such as cytochrome P450, in target tissues (Luu-The, 2001; Gilbert et al., 2002). The validity of this model awaits identification of additional enzymes that participate in *daf-12* ligand metabolism.

The *daf-9*-expressing head cells have been identified as XXXL/R where the *che-2* promoter is inactive (Ohkura et al., 2003). This is intriguing because expression of *daf-9* in ciliated sensory neurons using the *che-2* promoter was clearly able to suppress dauer arrest of *daf-9*(–) animals and hence substitute for the loss of *daf-9* activity in XXXL/R and other tissues. We obtained similar results when *daf-9* expression in ciliated sensory neurons was driven by the *osm-6* promoter (H.Y.M. and G.R., unpublished). Nevertheless, ectopic *daf-9* expression in mechanosensory neurons using the *mec-7* promoter did not suppress the dauer arrest phenotype of *daf-9*(–) animals (Gerisch and Antebi, 2004). To reconcile the different observations, we propose the following models. As XXXL/R cells are adjacent to the *che-2*-expressing, but not the *mec-7*-expressing, neurons (e.g. IL1s), one can imagine intercellular shuttling of lipophilic intermediates of hormone synthesis over a short distance. Alternatively, cytochrome P450 enzymes that normally act upstream and downstream of DAF-9 may be present in the ciliated neurons and given the cholesterol derived substrate is available, *daf-9* expression in these neurons may be sufficient to produce the bona fide hormone.

daf-9(+) activity in ciliated sensory neurons was sufficient to rescue the dauer arrest phenotype of *daf-9*-deficient animals, even though these animals display a gonadal migration defect. This phenocopies animals bearing *daf-9* weak loss-of-function alleles (Gerisch et al., 2001; Jia et al., 2002). Perhaps DAF-9 in ciliated sensory neurons can only produce a suboptimal dose of its cognate lipophilic hormone that is nevertheless sufficient to prevent dauer arrest. However, it is known that particular head chemosensory neurons emit key signals to control dauer arrest (Bargmann and Horvitz, 1991; Ren et al., 1996; Schackwitz et al., 1996), and they may be the target cells that respond to a paracrine signal generated by DAF-9. It is conceivable that *daf-9* may be involved in the synthesis of an endocrine signal for gonadal migration and a second paracrine signal for reproductive development. Accordingly, DAF-9 may be proximal in a hormone synthesis pathway in which the product of DAF-9 can be further modified by multiple downstream P450 enzymes to yield different lipophilic signaling molecules. This model predicts that the ciliated sensory neurons are unable to produce the endocrine signal that direct proper gonadal migration despite ectopic expression of *daf-9*, because of a lack of its downstream partners.

daf-9 may act in the pathway for the synthesis of a secondary signal that mediates the cell nonautonomous action of the *daf-2* insulin/IGF-I receptor and *daf-4* TGFβ type II receptor (Apfeld and Kenyon, 1998; Inoue and Thomas, 2000; Wolkow et al., 2000; Gerisch et al., 2001; Jia et al., 2002). In support of this hypothesis, we found that constitutive expression of *daf-9* in the hypodermis, under the control of a heterologous promoter, was sufficient to completely suppress the dauer arrest phenotype of *daf-7*(–) and *daf-1*(–) animals. The ability to bypass *daf-7*/TGFβ signaling deficiency by a gain-of-function *daf-9* transgene strongly suggests that *daf-9* is a major transducer of the *daf-7* reproductive signal.

Constitutive expression of *daf-9* in the hypodermis allowed partial suppression of the dauer arrest phenotype of *daf-2*(–) animals. It appeared that the dauer program was never initiated in a subset of tissues, while others were resistant to the excess hormonal signal generated by DAF-9. We postulate that *daf-9* may indeed mediate *daf-2* signaling in the hypodermis, pharynx and vulva. By contrast, it is unlikely to mediate *daf-2*(+) activity in the germline and intestine. One possibility is that *daf-2* may exert cell-autonomous action on the latter set of tissues and does not normally employ *daf-9* to relay its activity. Notably, mosaic analysis suggested that reproductive development of the germline may require additional *daf-2*(+)

activity that acts in a cell-autonomous manner (Apfeld and Kenyon, 1998). The partial dauer arrest phenotype displayed by *daf-2(e1370)* mutant animals overexpressing *daf-9* is similar to those observed in *daf-2(e1370)*; *daf-12(m20)* and *daf-2(e1370)*; *daf-12(m583)* animals (Larsen et al., 1995). The similarity in the effects of *daf-9* overexpression and *daf-12* severe loss of function, with respect to the highly tissue-specific phenotype of partial *daf-2* dauers, again highlights the close functional link between *daf-9* and *daf-12*. Nevertheless, the observation that *daf-2(e1370)*; *daf-12(m20)* animals lives twice as long as *daf-2(e1370)* animals provides an exception to this notion (Larsen et al., 1995), as the life span of *daf-2(e1370)* animals could not be altered by constitutive expression of *daf-9* in the hypodermis. It has been reported that the *daf-2* pathway acts in adult animals (up to 4 days old) to specify life span (Dillin et al., 2002); however, the activity of our hypodermal *daf-9* transgene ceases in 2-day-old adults. It may also be possible that *daf-2* and *daf-9* function independently to control adult life span.

We initially attempted to suppress dauer arrest of *daf-2*(–) and *daf-7*(–) animals by expressing *daf-9* under the control of its endogenous promoter. Unlike the hypodermis-specific *dpy-7* promoter, *daf-9* expressed from its own promoter failed to rescue *daf-2*(–) or *daf-7*(–) animals at the restrictive temperature. We note that the *daf-9* promoter is unable to support *daf-9* hypodermal expression in *daf-2*(–), *daf-7*(–) or natural dauers derived from starvation, even though *daf-9* expression persists in XXXL/R. This correlates well with our observations that *sdf-9* driven *daf-9* expression in XXXL/R alone is not sufficient to prevent dauer arrest of *daf-2*(–) and *daf-7*(–) animals. Taken together, we propose that favorable growth conditions, transduced in part by the *daf-2* and *daf-7* signaling pathways may trigger *daf-9* hypodermal expression at mid-L2 stage. Given the mass and coverage of the hypodermis across the entire animal, *daf-9* expression in this tissue may induce a dramatic increase in lipophilic hormone production that may be crucial in the initiation or reinforcement of the reproductive program. This is demonstrated by our results that constitutive expression of *daf-9* in the hypodermis is sufficient to promote reproductive development in dauer constitutive (Daf-c) mutant animals.

The execution of the dauer program is crucially dependent on *daf-12* (Antebi et al., 2000). Therefore, it is not surprising that its activity should be tightly regulated to prevent entry into diapause under favorable growth conditions. This is unlikely to be achieved through modulation of *daf-12* expression as it is expressed at high levels at the L2 stage in the hypodermis (Antebi et al., 2000). Instead, we propose that *daf-12* activity is regulated by the synthesis of a DAF-12 antagonist by DAF-9. According to this model, high level of *daf-12* expression and transcriptional activity will be counteracted by an increase in hypodermal *daf-9* expression that is *daf-12* dependent. However, attenuation or prevention of *daf-9* hypodermal expression may be a prerequisite for the dauer program, as seen in *daf-2*(–) and *daf-7*(–) animals. In this case, DAF-12 may disengage from the *daf-9* promoter. Alternatively, DAF-12 may repress *daf-9* hypodermal expression through recruitment of co-repressor complexes in response to dauer inducing signals.

A DAF-12 response element is located within intron 1 of the *daf-9*b isoform. This 181 bp sequence also appears to specify *daf-9* spermathecal expression independent of DAF-12. A nuclear receptor consensus half site (TGTTCT) is found within this sequence, which is also evident in an analogous location in the *C. briggsae daf-9* gene. Surprisingly, mutation of the nuclear receptor consensus binding site eliminated *daf-9* spermathecal expression without affecting the hypodermal expression. We propose that an unidentified nuclear receptor may bind to the TGTTCT element and regulate *daf-9* expression in the spermatheca. As the consensus binding site for DAF-12 is not known at present, we cannot exclude the possibility that DAF-12 may bind directly to other parts of the 181 bp intron 1. Alternatively, DAF-12 may be part of a transcriptional cascade and indirectly control *daf-9* hypodermal expression through other transcription factors.

Our results show that *daf-9* signals at a pivotal position in the dauer pathway to integrate *daf-2* insulin-like and *daf-7* TGFβ-like signaling pathways. The next challenge will be to identify the DAF-9 substrate that should shed light on the nature of the hormonal signal.

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