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SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in *C. elegans*

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SUMMARY

The dauer larva of the nematode *Caenorhabditis elegans* is a good model system for investigating the regulation of developmental fates by environmental cues. Here we show that SDF-9, a protein tyrosine phosphatase-like molecule, is involved in the regulation of dauer larva formation. The dauer larva of *sdf-9* mutants is different from a normal dauer larva but resembles the dauer-like larva of *daf-9* and *daf-12* dauer-constitutive mutants. Like these mutants, the dauer-constitutive phenotypes of *sdf-9* mutants were greatly enhanced by cholesterol deprivation. Epistasis analyses, together with the relationship between *sdf-9* mutations and *daf-9* expression, suggested that SDF-9 increases the activity of DAF-9 or helps the execution of the DAF-9 function. SDF-9 was expressed in two head cells in which DAF-9 is expressed. By their position and by genetic mosaic experiments, we identified these cells as XXXL/R cells, which are known as embryonic hypodermal cells and whose function at later stages is unknown. Killing of the *sdf-9*-expressing cells in the wild-type first-stage larva induced formation of the dauer-like larva. Since this study on SDF-9 and former studies on DAF-9 showed that the functions of these proteins are related to those of steroids, XXXL/R cells seem to play a key role in the metabolism or function of a steroid hormone(s) that acts in dauer regulation.

Supplemental data available on-line

Key words: *C. elegans*, Protein tyrosine phosphatase, Cholesterol, Dauer larva

INTRODUCTION

Environmental cues regulate various aspects of animal development. For example, sex determination in the atherinid fish is partly regulated by temperature (Conover and Kynard, 1981), and anti-juvenile hormones produced by some plants cause precocious metamorphosis in certain insects (Bowers et al., 1976). Such phenomena have attracted biologists' attention, because they show how animals adapt to their environment and how their phenotypes are determined by the interaction of their genome and environment. However, the mechanisms of such regulation mostly remain to be studied.

The dauer larva of the nematode *Caenorhabditis elegans* serves as a good model system for these issues. Under favorable conditions, *C. elegans* develops successively through four larval stages (L1-L4) to the reproductive adult in 3 days. However, under unfavorable conditions, it forms an alternative third-stage larva called dauer larva and arrest development. The dauer larva can survive for periods of several weeks or months without aging, while the adult animal can live only for about 2 weeks. When favorable conditions are encountered, the dauer larva begins to feed and resumes development to the adult stage (Riddle and Albert, 1997). It is known that the decision to enter the dauer stage is regulated by three environmental cues: food, temperature and a dauer-inducing pheromone that reflects population density (Golden and Riddle, 1982; Golden and Riddle, 1984a; Golden and Riddle, 1984b). At least two of them, food and dauer pheromone, are thought to be sensed by chemosensory neurons with ciliated endings directly exposed to the external environment (Albert et al., 1981; Perkins et al., 1986; Bargmann and Horvitz, 1991; Vowels and Thomas, 1992; Thomas et al., 1993; Schackwitz et al., 1996). The sensory neurons that participate in dauer formation have been identified by laser microsurgery. When sensory neurons ADF, ASG, ASI and ASJ are killed at the L1 stage, wild-type animals become dauer larvae and arrest development regardless of environmental conditions (Bargmann and Horvitz, 1991).

To identify genes involved in dauer signaling, many mutants that show abnormality in the dauer decision have been isolated (Riddle et al., 1981; Albert et al., 1981; Swanson and Riddle, 1981; Malone and Thomas, 1994; Inoue and Thomas, 2000). Such mutants, named *daf* (dauer formation abnormal), consist of two groups: dauer formation-constitutive (Daf-c) mutants, which enter the dauer stage even under conditions appropriate for reproductive growth, and dauer formation-defective (Daf-d) mutants, which develop as non-dauers even under harsh conditions. By epistasis analyses, these *daf* genes have been ordered into three pathways that act in parallel to regulate dauer

formation (Vowels and Thomas, 1992; Thomas et al., 1993; Gottlieb and Ruvkun, 1994). Molecular analyses revealed that these pathways correspond to cell-signaling pathways conserved among many animals, namely, cGMP (Coburn et al., 1998; Birnby et al., 2000), TGFβ (Georgi et al., 1990; Estevez et al., 1993; Schackwitz et al., 1996; Ren et al., 1996; Patterson et al., 1997; Inoue and Thomas, 2000) and insulin/IGF-I (Morris et al., 1996; Kimura et al., 1997; Ogg et al., 1997; Lin et al., 1997; Ogg and Ruvkun, 1998) pathways. The signals through the three pathways are integrated by a nuclear hormone receptor DAF-12, suggesting that the developmental decision is regulated by a lipophilic hormone(s) (Antebi et al., 2000; Snow and Larsen, 2000). Recently it was reported that one of the Daf-c genes, *daf-9*, encodes a cytochrome P450 and acts upstream of *daf-12*. The results indicate that DAF-9 is probably involved in the biosynthetic pathway for a ligand(s) of DAF-12 (Gerisch et al., 2001; Jia et al., 2002). *daf-9* mutants as well as special *daf-12* mutants that show a Daf-c phenotype form dauer-like larvae, which differ from normal dauer larvae in that their pharynx pumps and is not radially constricted. Furthermore, the Daf-c phenotypes resulting from weak alleles of these genes are greatly enhanced by cholesterol deprivation, consistent with the involvement of steroids in the function of *daf-12* and *daf-9*. *C. elegans* cannot synthesize sterols, and steroid hormones in *C. elegans* are considered to be made from cholesterol provided by culture media (Chitwood, 1999).

Conventional Daf-c genes contain mutations that cause highly penetrant Daf-c phenotypes. In addition, there are many other genes that, when mutated, result in only low-penetrance Daf-c, synthetic Daf-c (syn-Daf) and/or Hid (high temperature-induced dauer formation) phenotypes. Syn-Daf mutants and Hid mutants form dauer larvae only in a certain mutant background and at 27°C (a temperature too high for the reproduction of *C. elegans*), respectively (Bargmann et al., 1990; Avery, 1993; Katsura et al., 1994; Iwasaki et al., 1997; Prasad et al., 1998; Take-Uchi et al., 1998; Ailion et al., 1999; Sze et al., 2000; Sym et al., 2000; Daniels et al., 2000; Ailion and Thomas, 2000; Lanjuin and Sengupta, 2002). The presence of such genes suggests that the *daf* pathways may be bifurcated further or modified by other pathways. Analysis of these genes will help to elucidate the complicated regulatory network of dauer decision and provide a new insight into the environmental regulation of development.

Here we present genetic and molecular analyses of a new syn-Daf gene, *sdf-9*. It encodes a protein tyrosine phosphatase-like molecule, which seems to increase the activity of DAF-9 or help the execution of the DAF-9 function. The cells expressing *sdf-9* are the same as those expressing *daf-9* in the head and have been identified as XXXL/R cells in this study. These cells seem to play a key role in the metabolism of the steroid hormone(s) for DAF-12 that regulates dauer formation.

MATERIALS AND METHODS

Culture conditions and strains

Worms were grown by standard methods (Brenner, 1974; Sulston and Hodgkin, 1988). Normal NGM agar plates contained 5 μg/ml cholesterol, while cholesterol-poor NGM agar plates (NGM minus cholesterol) were the same but without the cholesterol. *Escherichia coli* strain OP50 was used as a food for worms. The following *C. elegans* strains were used in this work: wild-type N2, CB4856, *daf-2(e1370)*, *daf-3(e1376)*, *daf-5(e1386)*, *daf-7(e1372)*, *daf-9(e1406)*, *daf-12(m20)*, *daf-16(m27)*, *dpy-5(e61)*, *lon-2(e678)*, *rol-9(sc148)*, *sdf-9(ut157)*, *sdf-9(ut163)*, *sdf-9(ut169)*, *sdf-9(ut174)*, *sdf-9(ut187)*, *unc-31(e169)* and *unc-51(e369)*. The *sdf-9* mutants were backcrossed at least three times.

Dauer formation assays

Four to six adult hermaphrodites were allowed to lay eggs at 20°C for 4-7 hours on 6-cm NGM plates on which *E. coli* had been grown. Parent animals were then removed and the plates were incubated at the assay temperature. Dauer and non-dauer animals were counted after 3-3.5 days at 20°C, 2.5-3 days at 25°C, and 2-2.5 days at 27°C.

Molecular cloning of *sdf-9*

The *sdf-9* mutations were previously mapped to the right arm of chromosome V (N. Suzuki, T. Ishihara and I. Katsura, unpublished). More precise mapping was performed as follows. From the genotype *sdf-9(ut187)/unc-51(e369)rol-9(sc148)*, 12/12 Rol non-Unc recombinants were non-syn-Daf, and thus *sdf-9(ut187)* mapped near or on the right side of *rol-9*. Using SNPs (Wicks et al., 2001), *sdf-9(ut187)* was placed to the right of cosmid ZC15 (data not shown). Cosmids and PCR fragments from this region of the genome were introduced into *unc-31(e169); sdf-9(ut187)* animals, and the resulting transgenic lines were tested for the rescue of the Daf-c phenotype. A 14.8 kb PCR fragment containing the predicted ORFs Y44A6D.4 and Y44A6D.5 (*C. elegans* Sequencing Consortium, 1998) rescued the Daf-c phenotype. This fragment was digested with *Spe*I and *Nae*I, and the resulting 8.6 kb fragment containing Y44A6D.4 was subcloned into the *Spe*I-*Eco*RV site of pBluescript KS(+) (Stratagene Co.) to generate the plasmid pKO1. pKO1 was digested with *Eco*RV and *Hin*dIII, and the 5.7 kb fragment was cloned into the *Hin*cII-*Hin*dIII site of pBluescript KS(+) to generate the plasmid pKO2, which also rescued the Daf-c phenotype (data not shown).

Allele sequencing

Genomic DNA was isolated from the *sdf-9* mutant animals, and the *sdf-9* region was PCR-amplified. Mutation sites were determined by sequencing the mixed products of two independent PCR reactions.

cDNA cloning

The exon-intron structure of *sdf-9* gene was deduced by sequencing RT-PCR products amplified with gene-specific primers. The 5′ end of the gene was determined by sequencing PCR products obtained with SL1 leader sequence and gene-specific primers, while the 3′ end was determined by sequencing 3′ RACE products with gene-specific and artificial adapter primers.

Germline transformation

Germline transformation was carried out as described previously (Mello et al., 1991), at a concentration of 1-60 ng/μl test DNA with 5-15 ng/μl coinjection marker (*myo-3*::GFP, *gcy-10*::GFP, or pRF4(*rol-6*)) and 40-95 ng/μl carrier DNA (pBluescript KS(+)).

Reporter constructs

In this work, we used the modified Fire vectors pKOGzero and pKORzero as GFP and RFP (dsRed) vectors, respectively, for studying gene expression. pKOGzero was made by ligating fragments of three Fire vectors: a 2.9 kb *Xho*I-*Apa*I fragment of pPD95.67, a 98 base *Nco*I-*Xho*I fragment of pPD104.53 and a 1.6 kb *Xho*I-*Apa*I fragment of pPD104.91. pKORzero was made by substituting RFP cDNA for the GFP cDNA of pKOGzero. An 8.3-kb *C. elegans* genomic DNA containing the *sdf-9* gene was amplified with primers 5′-ATACGGAGCGCAAGGCTGTG-3′ and 5′-GGCTTTGGGATC-CACCACGGGCGGC-3′ and digested with *Hin*cII and *Bam*HI. The resulting 6.2 kb fragment was subcloned into the *Hin*cII-*Bam*HI site of pKOGzero to make pKOG6. pKOG6 was digested with *Kpn*I to

remove the nuclear localization signal, and self-ligated to generate pKOG7 (SDF-9::GFP; a 3.5 kb promoter and the entire coding region fused to GFP cDNA). pKOG6 was digested with *Hin*dIII and *Pvu*II and the 3.5 kb fragment was cloned into the *Hin*dIII-*Hin*cII site of pKOGzero and pKORzero to generate pKOG8 (*sdf-9p*::GFP1; a 3.5 kb promoter region fused to GFP cDNA) and pKOR1 (*sdf-9p*::RFP; a 3.5 kb promoter region fused to RFP cDNA). pKOG8 was digested with *Kpn*I to remove the nuclear localization signal and self-ligated to generate pKOG9 (*sdf-9p*::GFP2). The coding region (*Apa*I fragment) of the *sdf-9* gene from pKO1 was cloned into pBluescript KS(+) to generate pKO20 (*sdf-9* promoterless gene). The *daf-9* promoter region was amplified by PCR as described previously (Jia et al., 2002), and inserted into the *Pst*I site of pKO20 to generate pKO20-d9 (*daf-9p*::*sdf-9*(promoterless)). For DAF-9::GFP, a 7.7 kb PCR product from the *daf-9* genomic DNA containing a 5.4 kb 5′ upstream sequence and a 2.3 kb coding sequence was cloned into pKOGzero.

Mosaic analysis for the identification of *sdf-9*-expressing cells using cell-specific GFP markers

The following cell-specific GFP markers were used: *egl-4.a*::GFP for IL1VL/R (Fujiwara et al., 2002), *gcy-8*::GFP for AFDL/R (Yu et al., 1997), *gpa-4*::GFP for ASIL/R (Jansen et al., 1999), T23G5.5::GFP for CEPDL/R (Jayanthi et al., 1998) (T. Ishihara, unpublished) and *ttx-3*::GFP for AIYL/R (Hobert et al., 1997). *egl-4.a*::GFP, *gcy-8*::GFP, a mixture of *gpa-4*::GFP and *ttx-3*::GFP, and a mixture of *gpa-4*::GFP and *T23G5.5*::GFP, respectively, were injected into wild-type animals together with *sdf-9p*::RFP (pKOR1) to make animals that carry an extrachromosomal array containing one or two GFP markers and *sdf-9p*::RFP. The transgenic animals were grown on normal NGM plates to generate spontaneous mosaic animals, which were observed under a microscope at the L3 to adult stage. Mosaic animals were identified by the presence of *sdf-9p*::RFP in only one of the two *sdf-9*-expressing cells. Such animals were found at a frequency of several percent among animals showing RFP fluorescence, and examined for the presence or absence of each GFP marker, using both Nomarski and fluorescence optics.

Cell ablation

Laser microsurgery was carried out essentially as described previously (Bargmann and Avery, 1995). pKOG8 (*sdf-9p*::GFP1) was injected into *unc-31(e169)* animals together with *gcy-10*::GFP and pBluescript KS(+), and the established array was introduced into N2, *daf-3(e1376)*, *daf-16(m27)*, and *daf-12(m20)* animals. The cells expressing *sdf-9p*::GFP (XXXL/R cells, according to our assignment) were ablated in L1 larvae, which were then cultivated on NGM plates at 25°C. After 2 days, the numbers dauer larvae, dauer-like larvae and non-dauers were scored. The success of cell ablation was judged by the loss of *sdf-9p*::GFP fluorescence, and animals showing the fluorescence were ignored. Control animals, that were treated in the same way as the operated animals except that no cells were killed, did not form dauer or dauer-like larvae.

Expression of *sdf-9* under the control of the *daf-9* promoter

pKO20 (*sdf-9* promoterless gene) and pKO20-d9 (*daf-9p*::*sdf-9*(promoterless)), were injected into *unc-31(e169); sdf-9(ut187)* animals together with *gcy-10*::GFP and pBluescript KS(+), and the transgenic lines were tested for dauer formation.

Suppression of the Daf-c phenotype of various mutations by the wild-type *daf-9* transgene

A 9.3-kb PCR product from the *daf-9* genomic region containing 5.4-kb 5′ upstream sequence, 2.3-kb coding sequence, and 1.6-kb 3′ sequence was injected with *gcy-10*::GFP and pBluescript KS(+) into *unc-31(e169)*, *unc-31(e169);sdf-9(ut187)*, *daf-7(e1372)* and *daf-2(e1370)* animals, respectively. Multiple independent transgenic lines were tested for dauer formation. For the experiments with the *sdf-9(ut163)* mutant, the arrays were established in the *unc-31(e169)* background and introduced into *sdf-9(ut163)* animals by crossing. *sdf-9(ut187)*; Ex[*daf-9(+)*, *gcy-10*::GFP] animals were made from *unc-31(e169); sdf-9(ut187)*; Ex[*daf-9(+)*, *gcy-10*::GFP].

RESULTS

sdf-9 regulates dauer formation

Although the screen for single mutants with a strong Daf-c phenotype at 25°C is near saturation (Malone and Thomas, 1994), many syn-Daf mutants remain to be isolated (Avery, 1993; Katsura et al., 1994; Iwasaki et al., 1997; Take-Uchi et al., 1998; Ailion et al., 1999; Sym et al., 2000; Daniels et al., 2000; Ailion and Thomas, 2000; Lanjuin and Sengupta, 2002). To identify new genes that regulate dauer formation, we isolated 44 mutants showing Daf-c phenotypes in the *unc-31(e169)* mutant background and named them *sdf* (synthetic dauer formation abnormal; N.S., T.I. and I.K., unpublished). Five of them mapped in the same gene, which we named *sdf-9*. At 25°C, the *sdf-9* mutants exhibited a strong Daf-c phenotype in the *unc-31(e169)* background and a weak Daf-c phenotype in the wild-type background, while at 20°C, they showed a weak Daf-c or a Daf$^+$ phenotype even in the *unc-31(e169)* background (Tables 1, 2). The dauer larvae of the *unc-31; sdf-9* double mutants were abnormal in movement due to the *unc-31* mutation, but normal in morphology. However, the dauer larvae of the *sdf-9* single mutants had a pharynx that pumps and that does not show the radial constriction of the normal dauer pharynx (Fig. 1). In these aspects they resembled the dauer-like larvae of *daf-9* or *daf-12* Daf-c mutants (Albert and Riddle, 1988; Antebi et al., 1998; Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002), and hence we call them dauer-like larvae. The *sdf-9* dauer-like larvae recovered spontaneously and resumed development to fertile adults after 1-2 days. (78% (n=37) of *sdf-9(ut163)* dauer-like larvae recovered at 25°C, 94% (n=16) of *sdf-9(ut163)* and 100% (n=23) of *sdf-9(ut187)* dauer-like larvae, recovered at 20°C.) The adults of *sdf-9* had a normal gonads and vulvas, unlike *daf-9* adults. Although the *sdf-9* single mutants formed dauer-like larvae at 25°C under non-starving conditions, they formed normal dauer larvae under starving conditions.

Since some syn-Daf mutants show a strong Daf-c phenotype at 27°C (Hid) (Ailion and Thomas, 2000), we examined whether *sdf-9* mutants also show a Hid phenotype. Of the five *sdf-9* alleles, *ut163* produced a strong Hid phenotype, *ut157*, *ut169* and *ut187* weaker Hid than *unc-31(e169)* but stronger

Table 1. Dauer larva formation of *unc-31; sdf-9* double mutants

Strain	Dauer larva formation*	
	20°C	25°C
Wild type(N2)	0% (90)	0% (248)
unc-31(e169)	0% (201)	0% (164)
unc-31; sdf-9(ut157)	0.8% (130)	100% (149)
unc-31; sdf-9(ut163)	0% (133)	98.4% (188)
unc-31; sdf-9(ut169)	3.4% (117)	99.2% (118)
unc-31; sdf-9(ut174)	0% (107)	97.9% (142)
unc-31;sdf-9(ut187)	7.1% (141)	100% (165)

*The numbers in parentheses show the total numbers of animals scored.

Table 2. Dauer-like larva formation of *sdf-9* single mutants on NGM plates containing various concentrations of cholesterol

Dauer-like larva formation*	NGM plates			NGM minus cholesterol plates	
Strain	20°C	25°C	27°C†	20°C	25°C
Wild type(N2)	0% (554)	0% (701)	19.4% (676)	0% (439)	0% (319)
unc-31(e169)	ND‡	ND‡	99.3% (450)	0% (496)	0% (193)
sdf-9(ut157)	0% (451)	14.0% (563)	25.0% (468)	41.0% (227)	87.6% (177)
sdf-9(ut163)	0% (434)	35.1% (496)	77.3% (441)	2.2% (491)	97.5% (278)
sdf-9(ut169)	0% (319)	8.5% (520)	44.8% (495)	63.5% (189)	96.0% (149)
sdf-9(ut174)	0% (499)	0.8% (488)	4.5% (492)	12.4% (225)	79.9% (209)
sdf-9(ut187)	0% (549)	33.9% (360)	36.6% (481)	51.7% (323)	99.3% (280)

Dauer-like larva formation* at 27°C†	Cholesterol concentration			
Strain	5 μg/ml (normal NGM)	10 μg/ml	25 μg/ml	50 μg/ml
Wild type(N2)	1.1% (278)	0.9% (428)	0.2% (527)	0% (430)
sdf-9(ut163)	75.4% (337)	49.1% (334)	23.1% (412)	49.4% (385)
sdf-9(ut187)	45.7% (243)	4.4% (387)	2.1% (423)	3.0% (335)

*The numbers in parentheses show the numbers of animals scored.
†The data at 27°C include both dauer-like larvae and normal dauer larvae.
‡Not determined
The reason for the considerably different results between *ut169* and *ut174*, whose mutations turned out to be identical by DNA sequencing, is unknown.

than the wild type (Table 2). Such allele-specific Hid phenotypes have been reported for *tax-2* mutants (Ailion and Thomas, 2000).

Position of *sdf-9* gene in the dauer pathway

To determine the position of the *sdf-9* gene in the dauer pathway, we carried out genetic epistasis experiments. Under reproductive growth conditions, the *sdf-9(ut163)* single mutant formed dauer-like larvae at 25°C. The *daf-16(m27)* and *daf-12(m20)* mutations suppressed this phenotype, while *daf-3(e1376)* enhanced it (Table 3). The presence of the *daf-5(e1386)* mutation had only a very small effect on the Daf-c phenotype of *sdf-9*: N2, *daf-5*, *ut163* and *daf-5;ut163* mutants produced 0% (n=236), 0% (n=416), 80% (n=256), and 52% (n=256) dauer and dauer-like larvae, respectively, at 25.5°C on NGM minus cholesterol. We also tested suppression of the Daf-c phenotype of *unc-31(e169); sdf-9* at 20°C, using three *sdf-9* alleles, *ut163*, *ut174* and *ut187*. The results were essentially the same as the suppression of *sdf-9(ut163)*, i.e., *daf-16* and *daf-12* mutations suppressed the *unc-31; sdf-9* Daf-c phenotypes, while *daf-3* enhanced it (data not shown). The results indicate that *sdf-9* acts upstream of *daf-16* and *daf-12* but downstream of or in parallel with *daf-3* and *daf-5*. Unidentified interaction may exist between *daf-3* and *sdf-9*,

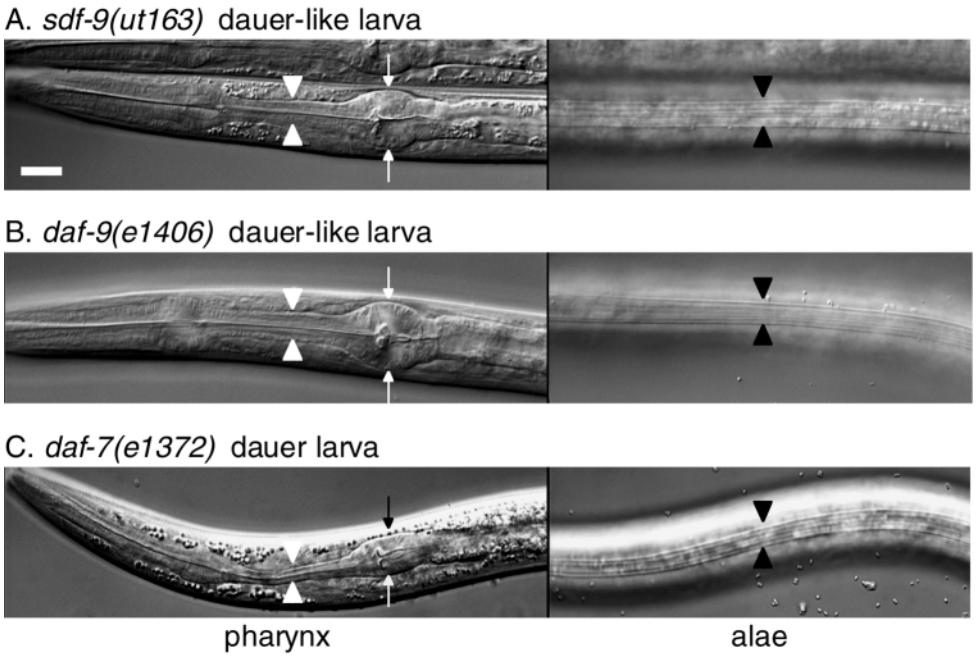

Fig. 1. Nomarski micrographs of the pharynx and the lateral cuticle of mutant dauer larvae. (A) *sdf-9(ut163)* dauer-like larva. (B) *daf-9(e1406)* dauer-like larva. (C) *daf-7(e1372)* dauer larva. Left and right panels show the pharynx and the alae, respectively. In the left panels the arrows indicate the terminal bulb and the arrowheads the isthmus. The pharynxes of *sdf-9* and *daf-9* mutants are similar to the L3 pharynx and thicker than the *daf-7* dauer pharynx, which looks like the pharynx of the wild-type dauer larva. In addition to pharyngeal morphology, the pharynx of normal dauer larvae (e.g. wild type, *daf-7*, and *daf-2*) never pumps, whereas those of *sdf-9* and *daf-9* dauer larvae pump. The arrowheads in the right panels indicate longitudinal ridges called alae. They are present at the L1, dauer, and adult stages but absent at the L3 stage. Scale bar: 10 μm.

Table 3. Dauer formation at 25°C of various mutants without and after killing XXXL/R cells

Strain*	Dauer-like larvae	Dauer larvae	$n^{\dagger}$
Without killing XXXL/R cells			
Wild type(N2)	0%	0%	370
sdf-9(ut163)	42.2%	0%	415
daf-3(e1376)	0%	0%	440
daf-16(m27)	0%	0%	293
daf-12(m20)	0%	0%	310
sdf-9; daf-3	76.6%	0%	312
daf-16; sdf-9	0%	0%	347
sdf-9; daf-12	0%	0%	341
After killing XXXL/R cells			
Wild type(N2)	31.4%	2.3%	86
unc-31(e169)	0%	59.5%	37
daf-3(e1376)	65.9%	0%	44
daf-16(m27)	0%	0%	53
daf-12(m20)	0%	0%	40
Mock kill (control)			
Wild type(N2)	0%	0%	24
unc-31(e169)	0%	0%	22
daf-3(e1376)	0%	0%	24
daf-16(m27)	0%	0%	7
daf-12(m20)	0%	0%	17

*All the strains used for the laser surgery experiments carry the extrachromosomal array, *Ex[sdf-9p*::*GFP1*(pKOG8), *gcy-10*::GFP*]*.
†Total number of animals.

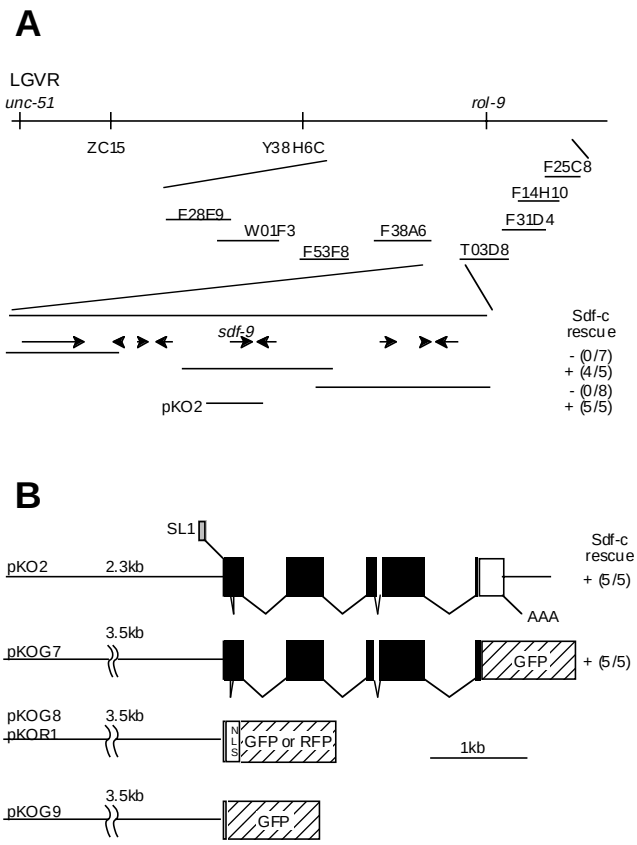

Fig. 2. Molecular cloning of *sdf-9*. (A) Genetic and physical map of the *sdf-9* region. The arrows indicate genes predicted by the *C. elegans* genome project. Genomic DNA fragments containing the predicted gene Y44A6D.4 rescued the Daf-c phenotype of *unc-31(e169); sdf-9(ut187)* double mutants. (B) Structure of *sdf-9* gene and reporter constructs. Boxes represent exons. pKO2 and pKOG7 rescued the *unc-31(e169); sdf-9(ut187)* Daf-c phenotype.

because the *daf-3(e1376)* mutation slightly enhanced the Daf-c phenotype of *sdf-9(ut163)*, as in the case of *daf-3(mgDf90)* and *daf-12(rh273daf-c)* (Gerisch et al., 2001).

sdf-9 encodes a protein tyrosine phosphatase-like molecule

We found that the *sdf-9* gene corresponds to the predicted ORF Y44A6D.4 by three-factor cross, SNP mapping, and rescue experiments (Fig. 2 and Materials and Methods). The cDNA analysis showed that it has the SL1 trans-splice leader sequence, that it encodes a protein of 345 amino acids, and that the prediction of exons was correct except for the 3′ region (Fig. 3).

Blast search revealed that the predicted SDF-9 protein is homologous to protein tyrosine phosphatases (PTPs; Fig. 4). PTPs form a family of diverse proteins characterized by the presence of at least one conserved PTP domain (240-250 amino acids) (Tonks and Neel, 2001). SDF-9 is a non-transmembrane PTP that has only one PTP domain and no other known domains. The PTP domain normally contains the active site motif HCxxxxxR (where x represents any amino acid), but the corresponding sequence of SDF-9 was QSARGSSR. Since the cysteine residue in the active site is essential for the activity of some PTPs (Streuli et al., 1989; Streuli et al., 1990; Guan and Dixon, 1990; Guan et al., 1991; Zhou et al., 1994), SDF-9 may not have the activity of protein tyrosine phosphatase.

The coding regions and exon-intron boundaries of the five *sdf-9* alleles were sequenced to identify mutation sites. The *ut163* mutation was a C to T transition resulting in the change of Pro 175 to Leu. The *ut157* and *ut187* mutations were a G to A transition that changed the conserved Arg 264 to Lys. The *ut169* and *ut174* mutations were a G to A transition, and the TGG codon for Trp 169 was replaced by a stop codon, TGA. This nonsense mutation would result in a truncated protein lacking the PTP active site and hence these alleles are likely to be null.

Expression pattern of *sdf-9*

We investigated the expression pattern of *sdf-9* using GFP and RFP fusion genes. pKOG7 (SDF-9::GFP) rescued the Daf-c phenotype of *unc-31(e169); sdf-9(ut187)* (data not shown), indicating that the GFP fusion protein is functional and that the place and the time of this expression are sufficient for normal dauer regulation. The expression of SDF-9::GFP was observed from late embryos to adults, and primarily in two cells anterior to the nerve ring. The SDF-9::GFP fusion protein was localized in the cytoplasm. The fluorescence intensity was essentially constant from the threefold stage of embryos to L4 larvae, but slightly weaker at the adult stage. To analyze the cell shape, a promoter-fusion GFP construct, *sdf-9p*::GFP2, was introduced into wild-type animals. Like SDF-9::GFP, *sdf-9p*::GFP2 was expressed strongly in two ventral cells anterior to the nerve ring (Fig. 5A), but additional faint signals were occasionally observed in several neurons posterior to the nerve ring. The cells expressing *sdf-9p*::GFP2 and SDF-9::GFP had two (or sometimes three) short processes, which had a complex shape

and looked different from those of sensory neurons. In some transgenic animals, one of the processes occasionally extended to the anterior tip of the head (Fig. 5B). In dauer larvae, one of the processes was long and extended posteriorly, but did not seem to enter the nerve ring (Fig. 5C). The positions of the two cells were variable, with the right-hand cell occasionally found anterior to the metacorpus (19%, n=508).

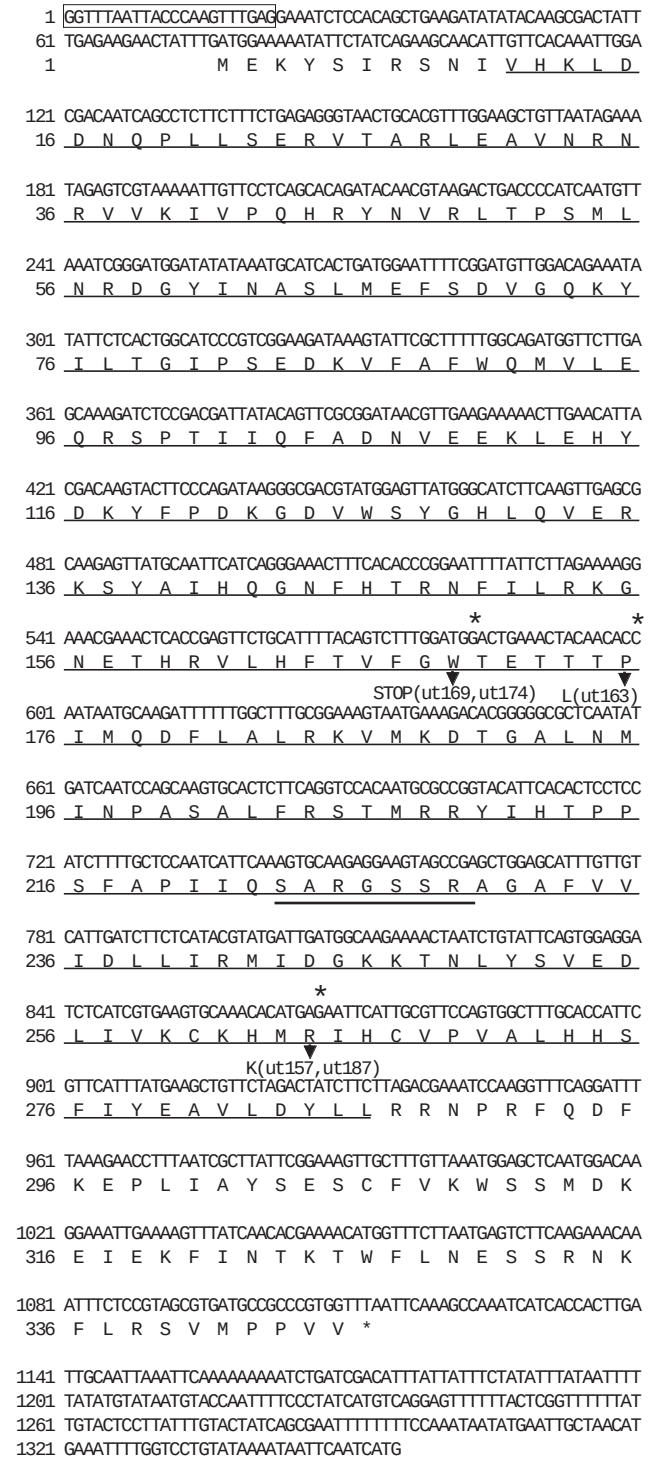

Fig. 3. cDNA and deduced amino acid sequence of *sdf-9*. The SL1 trans-splice leader sequence at the 5′ end is boxed. The PTP domain is underlined and the active site sequence is doubly underlined. The DNA bases and the amino acid residues that are changed in the *sdf-9* mutants are marked with asterisks and arrowheads, respectively.

These results were similar to the expression pattern of *daf-9* in the head region (Jia et al., 2002). We therefore made transgenic animals carrying both *sdf-9p*::RFP and DAF-9::GFP, and compared the expression of the two fusion genes. As shown in Fig. 6A, the cells expressing *sdf-9p*::RFP were the same as those expressing DAF-9::GFP in the head.

These cells were identified tentatively as IL1VL/R or URAVL/R in other laboratories, mainly on the basis of their positions (Gerisch et al., 2001; Jia et al., 2002). However, since it is difficult to identify cells anterior to the nerve ring only by their positions (Bargmann and Avery, 1995), we ascertained the identification of these cells by a newly designed genetic mosaic experiment using known cell-specific markers, as follows.

Identification of *sdf-9*-expressing cells

We first chose the candidates of the *sdf-9*-expressing cells (Fig. 7) by their positions, using figure 2 of White et al. (White et al., 1986). Then, ciliated sensory neurons (hatched in Fig. 7) were excluded from the candidates, because we found that the cells expressing *sdf-9p*::RFP did not express *che-2p*::GFP (data not shown), which is known to be expressed in most ciliated sensory neurons including those shown in Fig. 7 (Fujiwara et al., 1999).

We then carried out mosaic analysis using *sdf-9p*::RFP and some cell-specific GFP markers (Table 4). When a mixture of two DNA clones is injected into germline cells of *C. elegans*, they join together and form an extrachromosomal array containing both DNAs (Mello et al., 1991), which is inherited relatively stably but lost at a certain probability by segregation through cell division (Miller et al., 1996). The rationale of this mosaic analysis is that the co-loss or co-presence of GFP fluorescence and RFP fluorescence in mosaic animals should correlate with the degree of lineal relatedness between the GFP- and RFP-expressing cells. Fig. 6B shows the cell lineage of the GFP marker cells and the candidates of *sdf-9*-expressing cells (white cell bodies in Fig. 7), which was used as a reference in this experiment.

The results (Table 4) indicate, for instance, that the left-hand cell that expresses *sdf-9*::RFP (abbreviated as *sdf-9*L below) is nearer to ASIL (88% co-loss or co-presence) and ASIR (80%) in the cell lineage than the right-hand cell that expresses *sdf-9*::RFP (*sdf-9*R) (12% and 20%, respectively). However, *sdf-9*L is nearer to AFDR (55%) and CEPDL (89%) in the cell lineage than *sdf-9*R (45% and 11%, respectively), while *sdf-9*R is nearer to AFDL (64%) and CEPDR (79%) than *sdf-9*L (36% and 21%, respectively). These and other results are summarized as the cell lineages shown in Fig. 6C, which shows that *sdf-9*R is derived from the ABa blastomere, while *sdf-9*L is from the ABp blastomere. The cells that satisfy this condition are limited to XXXL/R among the candidate cells shown in Fig. 6B.

There is additional evidence supporting the proposal that the *sdf-9*::RFP-expressing cells are XXXL/R. If we assume that *sdf-9*L/R are XXXL/R, as many as 94% of the mosaic animals could be explained by a single loss of the extrachromosomal array, and only 6% of the mosaic animals require two losses and none require three losses, for their formation (see supplemental data at http:/dev.biologists.org/supplemental/). In

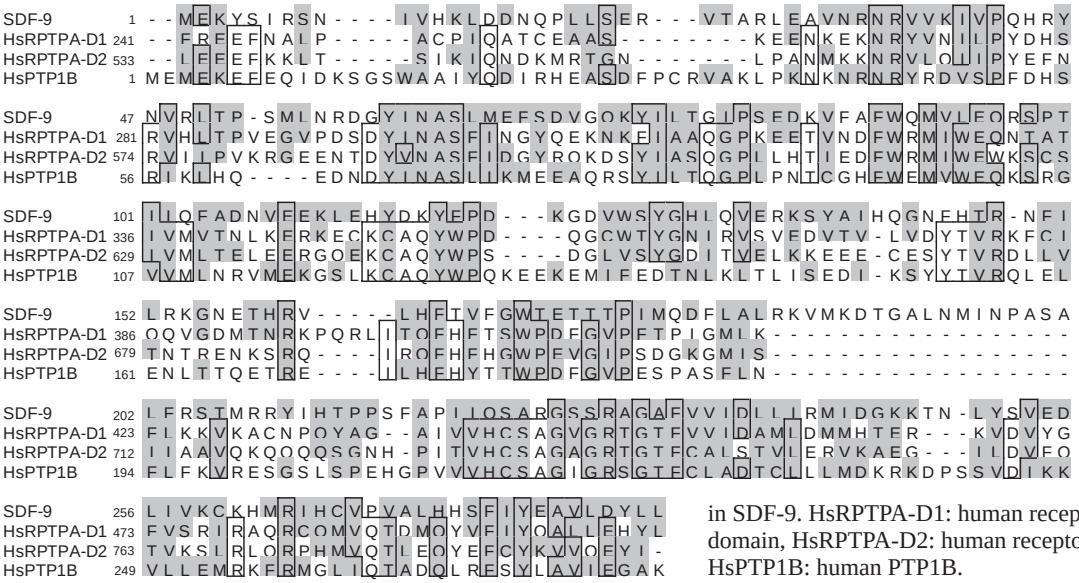

Fig. 4. Alignment of the PTP domains of SDF-9 and known PTPs. Conserved residues and homologous residues (gray) are boxed. The active site of PTP is underlined. The cysteine residue (Cys 223) that is essential for phosphatase activity is replaced by serine in SDF-9. HsRPTPA-D1: human receptor PTP alpha proximal domain, HsRPTPA-D2: human receptor PTP alpha distal domain, HsPTP1B: human PTP1B.

contrast, if *sdf-9*L/R were any of the other cells shown in Fig. 6B, much higher percentages of mosaic animals would have suffered multiple losses (see Table S3 at http:/dev.biologists.org/supplemental/). Since multiple losses of the extrachromosomal array in one animal do not occur so frequently, the results support the above conclusion.

Killing of XXXL/R cells induces the formation of dauer-like larvae

To determine whether the *sdf-9*-expressing cells, which we identified as XXXL/R cells, are involved in dauer formation, we killed them in L1 larvae by laser microbeam surgery. At 25°C, about 30% of the operated wild-type animals became dauer-like larvae resembling those of *sdf-9* and having a non-constricted, pumping pharynx (Table 3). Furthermore, about 60% of the operated *unc-31(e169)* animals formed Unc but otherwise normal dauer larvae that resemble *unc-31; sdf-9* dauer larvae (Table 3). We also killed these cells in some Daf-d mutants: *daf-3(e1376)*, *daf-16(m27)* and *daf-12(m20)*. When the XXXL/R cells were killed, *daf-3* animals formed dauer-like larvae, whereas *daf-16* and *daf-12* animals formed neither dauer nor dauer-like larvae (Table 3). These results suggest that XXXL/R cells are important in preventing formation of dauer-like larvae and that the position of their function in the dauer pathway is the same as that of *sdf-9*.

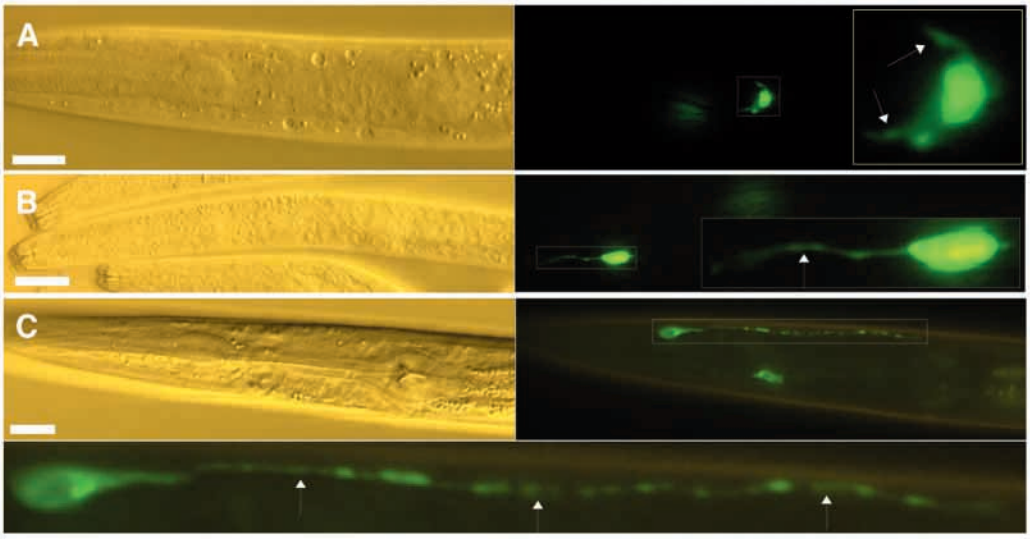

Fig. 5. Expression pattern of *sdf-9*. (A) Expression of *sdf-9p*::*GFP2* (pKOG9) in the head region of an L3 larva. The left-hand cell, which is in focus in this micrograph, is strongly fluorescent, while the right-hand cell, which is also strongly fluorescent, is out of focus. The arrows indicate short dendrite-like structures. (B) Expression of *sdf-9p*::*GFP2* expression in the head region of an L1 larva. The right-hand cell that express the GFP fusion gene is indicated. The arrow shows the dendrite-like process, which extends to the tip of the head. (C) Expression of *SDF-9*::*GFP* (pKOG7) in two head cells of a *daf-9(e1406)* dauer larva. One of the two dendrite-like processes extends posteriorly. Both cells have processes, but those of the left-hand cell are out of focus. The images in the right white boxes in (A) and (B) are magnified versions of those in the left white boxes, respectively, and the bottom image is a magnified version of that in the white box in (C). Scale bar: 10 μm.

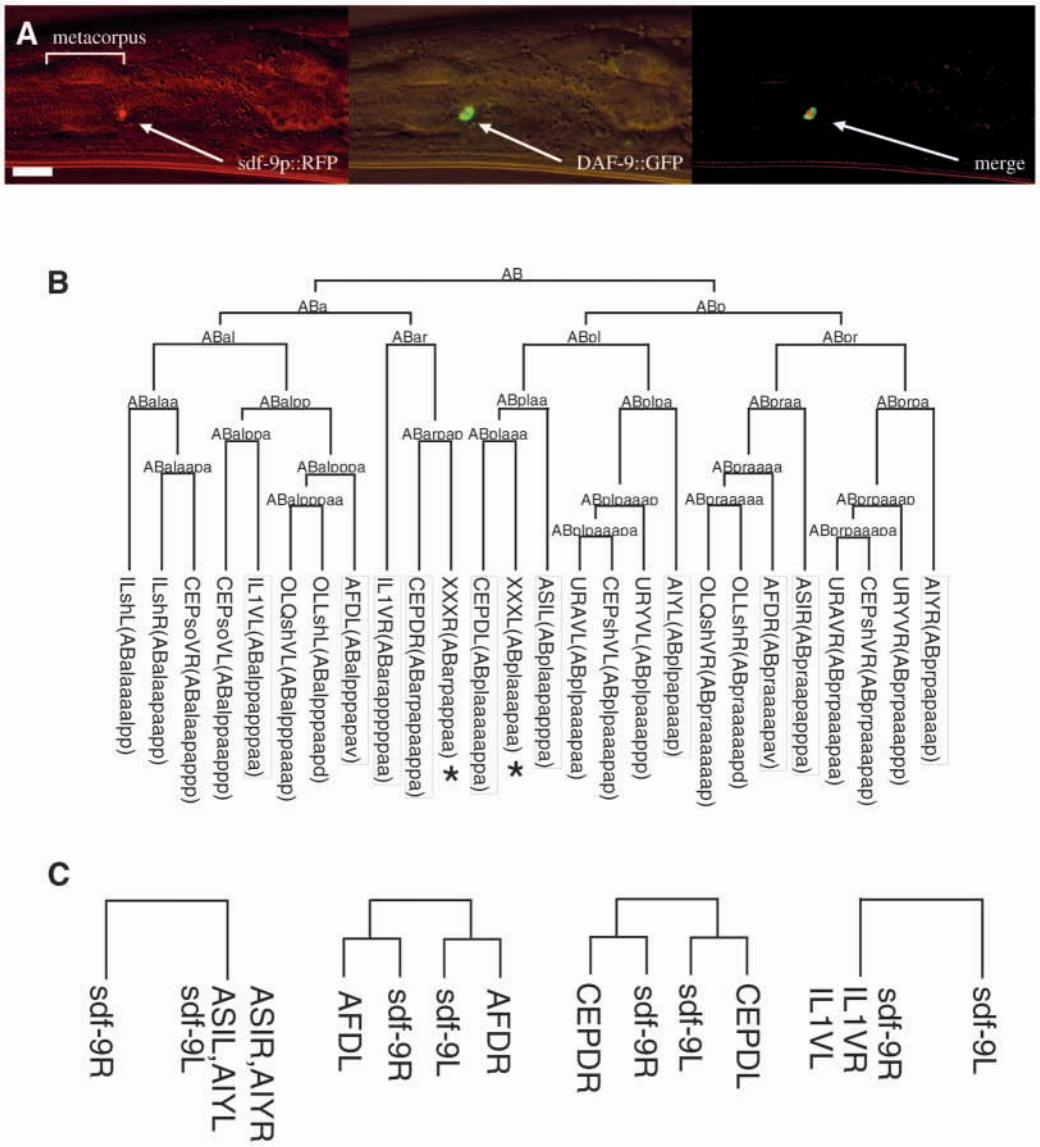

Fig. 6. Identification of the *sdf-9*-expressing cells. (A) Expression of *sdf-9p*::*RFP* (left) and *DAF-9*::*GFP* (middle) in an adult animal. Only the left-hand cell is in focus. A merged image (right) shows that RFP (nuclear) and GFP (perinuclear) are expressed in the same cell. Scale bar: 10 μm. (B) Cell lineage of the candidates of *sdf-9*-expressing cells (not boxed) and the neurons that express the GFP markers (boxed). The candidates are selected by their positions in the head and by the absence of *che-2* expression (Fig. 7). Mosaic experiments indicated that *sdf-9*-expressing cells are XXXL and XXXR, which are indicated by asterisks. a: anterior, p: posterior, d: dorsal, v: ventral, l: left, r: right. (C) Cell lineages of the cells expressing *sdf-9*::RFP and the GFP marker cells as deduced from the mosaic experiments. Only the topology but not the left-right relationship in these lineages is relevant. The sublineages of five cells in the most left lineage and three cells in the most right lineage could not be determined from the data shown in Table 4.

Expression of *sdf-9* gene under the control of the *daf-9* or *npc-1* promoter rescues the Daf-c phenotype of *unc-31; sdf-9*

Because *sdf-9* is expressed in the *daf-9*-expressing cells in the head, we expressed the *sdf-9* gene under the control of the *daf-9* promoter and tested whether this is sufficient for the wild-type phenotype of *sdf-9* gene. The Daf-c phenotype of the *unc-31(e169); sdf-9(ut187)* double mutant was suppressed by the *sdf-9* gene under the control of a *daf-9* promoter (see Table S4 at http:/dev.biologists.org/supplemental/), but not by the promoterless *sdf-9* gene (data not shown). The result indicates that the expression driven by the *daf-9* promoter is sufficient for the function of the *sdf-9* gene in normal dauer regulation. By using GFP and RFP fusion genes, we already showed that the cells in which *sdf-9* is strongly expressed are a subset of cells in which *daf-9* is strongly expressed. The result in this section confirms this conclusion through the function of *sdf-9*.

We also expressed *sdf-9* under the control of other promoters. *npc-1* and *npc-2* are homologs of the Niemann-Pick type C disease gene, which plays a role in intracellular sterol transport (Hoekstra and van IJzendoorn, 2000; Ioannou, 2001). In *C. elegans*, it is known that the double mutant *npc-2; npc-1* shows a Daf-c phenotype (Sym et al., 2000). We found that *npc-1p*::GFP was expressed in the *sdf-9*-expressing cells. Furthermore, the expression of *sdf-9* under the control of the *npc-1* promoter rescued the Daf-c phenotype of *unc-31(e169); sdf-9(ut187)* (data not shown). These results suggest that the *sdf-9*-expressing cells, which we identified as XXXL/R, may be important in the metabolism of steroids or steroid hormone signaling.

Cholesterol deprivation enhances the weak Daf-c phenotype of *sdf-9* mutants

It was reported that cholesterol deprivation enhances the weak Daf-c phenotype of *daf-9(rh50)* and *daf-12(rh284)* mutants (Gerisch et al., 2001). Since *sdf-9* resembles *daf-9* in dauer-like larvae and expression pattern, we investigated the dauer formation of *sdf-9* mutants on NGM plates without a supplement of cholesterol (NGM minus cholesterol). The *sdf-9* Daf-c phenotype was enhanced by cholesterol deprivation (Table 2).

We confirmed that a functional SDF-9::GFP (pKOG7) rescued this Daf-c phenotype of the *sdf-9(ut163)* mutant (see Table S5 at http:/dev.biologists.org/supplemental/). We also found that increased concentrations of cholesterol tend to suppress the Daf-c phenotype of *sdf-9* mutants (Table 2). These results show that cholesterol or its metabolite regulates dauer formation in cooperation with the regulatory pathway including the *sdf-9* gene.

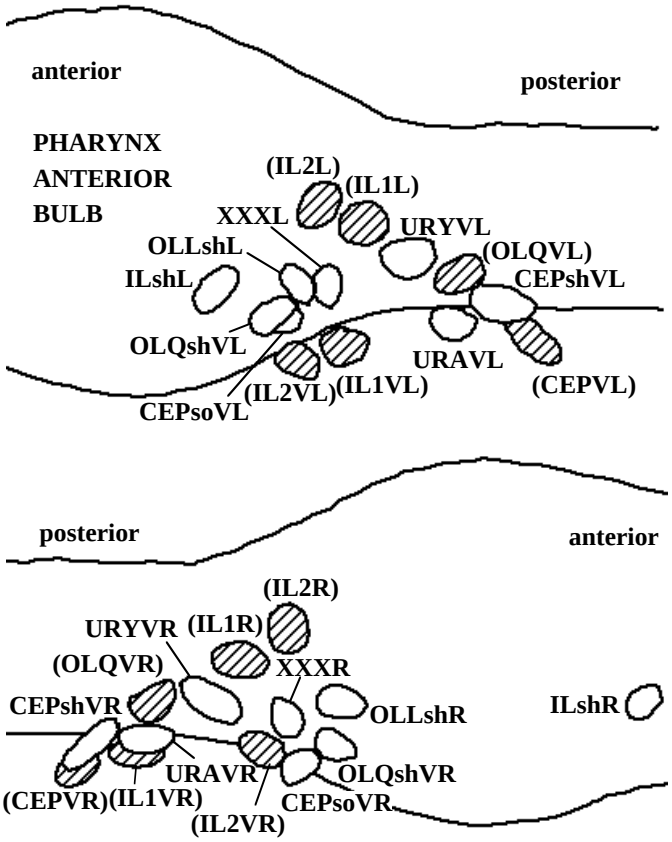

Fig. 7. Candidates of the *sdf-9*-expressing cells considered in the mosaic experiment [modified from figure 2 by White et al. (White et al.,1986)]. The figures show the cell bodies on the ventral side near the anterior bulb (metacorpus) of the pharynx. The top and the bottom diagrams show the left-hand and the right-hand views, respectively. Hatched cells are reported to express *che-2*::GFP (Fujiwara et al., 1999), and hence are excluded from the list of candidates.

The Daf-c phenotype of *sdf-9* single and *unc-31; sdf-9* double mutants is suppressed by the overexpression of the wild-type *daf-9* gene

We examined the expression of DAF-9::GFP in the wild type and the following mutant backgrounds: *unc-31(e169), sdf-9(ut174), sdf-9(ut187), unc-31(e169); sdf-9(ut174)*, and *unc-31(e169); sdf-9(ut187)*. The expression did not change in these mutant backgrounds (data not shown), but the transgene weakly suppressed the Daf-c phenotype of *unc-31; sdf-9* mutants, although it weakly enhanced the dauer formation of the *unc-31* mutant (data not shown). We therefore examined whether the wild-type *daf-9* gene suppresses the Daf-c phenotypes of *sdf-9* single mutants on NGM minus cholesterol and *unc-31; sdf-9* double mutants on NGM. As shown in Table 5, the extrachromosomal array of the wild-type *daf-9* gene strongly suppressed the Daf-c phenotypes of *sdf-9(ut163), sdf-9(ut187)* and *unc-31(e169); sdf-9(ut187)*, but not those of *daf-7(e1372)* and *daf-2(e1370)*, in which *daf-9p*::GFP was also expressed (data not shown). These results suggest that SDF-9 enhances the function but not the expression of DAF-9.

DISCUSSION

sdf-9, a new syn-Daf gene, encodes a PTP-like molecule

The *sdf-9* gene was identified first by the syn-Daf phenotype of its mutants. Then we cloned the gene by mapping and mutant rescue experiments. The predicted amino acid sequence revealed that the protein product of *sdf-9* has homology to protein tyrosine phosphatases (PTPs) but that the active site cysteine of PTP is missing in SDF-9. This cysteine residue forms a bond with a phosphate group in a reaction intermediate (Guan and Dixon, 1991), and many mutant PTPs in which this cysteine is replaced by serine have no detectable phosphatase activity (Streuli et al., 1989; Streuli et al., 1990; Guan and Dixon, 1990; Guan and Dixon, 1991; Zhou et al., 1994). These mutant PTPs, however, have the activity of binding to phosphorylated substrates (Jia et al., 1995). Many organisms including *C. elegans*, *Drosophila*, yeast and humans have such PTP-like molecules as wild-type proteins. These proteins may

Table 4. Mosaic analysis for the identification of *sdf-9*-expressing cells

	gpa-4::GFP ASI		*ttx-3*::GFP AIY		*gcy-8*::GFP AFD		T23G5.5::GFP CEPD		*egl-4.a*::GFP IL1V	
GFP marker marked cells	L	R	L	R	L	R	L	R	L	R
Mosaic animals with only left-hand *sdf-9*::RFP										
(1) Animals with marker GFP	17	16	18	18	6	8	12	6	8	8
(2) Animals without marker GFP	0	1	1	1	4	2	0	6	3	3
(3) Total	17	17	19	19	10	10	12	12	11	11
Mosaic animals with only right-hand *sdf-9*::RFP										
(4) Animals with marker GFP	3	4	4	5	10	8	3	16	16	16
(5) Animals without marker GFP	5	4	3	2	2	4	13	0	0	0
(6) Total	8	8	7	7	12	12	16	16	16	16
Linkage with left-hand *sdf-9*::RFP {(1)+(5)}/{(3)+(6)}	88%	80%	81%	77%	36%	55%	89%	21%	30%	30%
Linkage with right-hand *sdf-9*::RFP {(2)+(4)}/{(3)+(6)}	12%	20%	19%	23%	64%	45%	11%	79%	70%	70%

Table 5. Suppression of the Daf-c phenotype by *daf-9(+)* transgene

Strain†	Dauer(-like) larva formation at 25°C*	
	+Ex‡	–Ex§
sdf-9(ut163)¶		
line 1	0.9% (112)	87.7% (204)
line 2	0% (102)	81.3% (198)
sdf-9(ut187)¶		
line 1	2.0% (50)	85.4% (48)
line 2	2.2% (134)	65.5% (165)
*unc-31(e169); sdf-9(ut187)***		
line 1	0% (29)	90.5% (84)
line 2	32.7% (55)	92.4% (66)
*daf-7(e1372)***		
line 1	85.1% (47)	100% (57)
line 2	97.1% (35)	100% (86)
*daf-2(e1370)***		
line 1	100% (49)	100% (35)
line 2	87.8% (41)	100% (41)

*The numbers in parentheses show the numbers of animals scored.
†The strains carry the extrachromosomal array, *Ex[daf-9(+), gcy-10::GFP]*.
‡Animals carrying the extrachromosomal array.
§Animals that had lost the extrachomosomal array.
¶These strains were grown on NGM minus cholesterol and dauer-like larvae were scored.
**These strains were grown on normal NGM and dauer larvae were scored.

function as novel regulatory factors in signal transduction systems involving protein tyrosine phosphorylation, by binding to phosphorylated tyrosine residues in proteins (Wishart et al., 1995; Wishart and Dixon, 1998). Alternatively, these PTP-like molecules may have the activity of protein tyrosine phosphatase, but their correct substrates have not been found. In any case, the results of this study suggest that dauer regulation involves a regulatory system containing a tyrosine-phosphorylated protein.

SDF-9 enhances the DAF-9 function

The results of this study showed that the function of *sdf-9* is closely related to that of *daf-9*. First, like *daf-9* mutants, *sdf-9* mutants form dauer-like larvae under reproductive growth conditions, and normal dauer larvae under dauer-inducing conditions. Moreover, like *daf-9*, the formation of dauer-like larvae by *sdf-9* mutants is enhanced by cholesterol deprivation. Second, *sdf-9* is expressed in the two head cells in which *daf-9* is expressed. Third, like the Daf-c phenotype of *daf-9*, the Daf-c and syn-Daf phenotypes of *sdf-9* are suppressed by *daf-12* but not by *daf-3*.

In some aspects, however, *sdf-9* mutants show weaker phenotypes than *daf-9* mutants, or the *sdf-9* gene has only a part of the properties of *daf-9* gene. First, even a putative null mutant of *sdf-9* shows only a weak, temperature-sensitive Daf-c phenotype, while many *daf-9* mutants show strong, non-conditional Daf-c phenotypes. Second, the formation of dauer-like larvae is suppressed by *daf-16* in the case of *sdf-9* mutants but not in *daf-9* mutants. Third, *sdf-9* is expressed essentially only in two cells in the head, while *daf-9* is expressed in hypodermis, vulval blast cells and spermatheca at some stages of development, besides the two head cells. Fourth, *sdf-9* mutants seem to show normal longevity, at least at 25°C (K.O., T.I. and I.K., unpublished), while *daf-9* mutants show increased longevity under certain conditions (Gerisch et al., 2001; Jia et al., 2002). These results may be explained, if the *sdf-9* function is partially redundant, if *sdf-9* controls only part of the multiple functions of *daf-9*, or if *sdf-9* just enhances the *daf-9* function.

The functional relationship between *sdf-9* and *daf-9* can be further speculated on. This study revealed that the Daf-c and syn-Daf phenotypes of *sdf-9* are suppressed by the overproduction of the wild-type *daf-9* gene, and that DAF-9::GFP is expressed even in the *sdf-9* mutant background. The results show that SDF-9 enhances the function but not the synthesis of DAF-9. SDF-9 may increase the activity of the DAF-9 P450 molecule or may help the execution of the DAF-9 function, for instance, through the metabolism or transport of the substrate or product of DAF-9.

Position of *sdf-9* in the dauer regulatory pathway

Although we have discussed that *sdf-9* is closely related to *daf-9* in its function, the position of the *sdf-9* gene in the dauer regulatory pathway is yet another issue to be discussed. The Daf-c phenotypes of *sdf-9* as well as *daf-9* were suppressed by *daf-12* but not by *daf-3* or *daf-5*. These results indicate that the *sdf-9* gene, like *daf-9*, acts upstream of *daf-12*, but downstream of or in parallel with the TGFβ pathway genes *daf-3* and *daf-5*. Since the *sdf-9* Daf-c phenotype is not enhanced by *daf-5*, its enhancement by *daf-3* is probably related to the Daf-c phenotype of *daf-3* (but not *daf-5*) at 27°C (Ailion and Thomas, 2000).

Unlike *daf-9*, the Daf-c phenotypes of *sdf-9* mutations were suppressed by *daf-16*. This result can be interpreted in the light of the laser microsurgery experiments. The dauer larva formation induced by the ablation of XXXL/R showed the same suppression pattern as that of *sdf-9* mutants. Furthermore, like *sdf-9* mutations, the operated larvae produced mainly dauer-like larvae in the wild-type background, and normal dauer larvae in the *unc-31* background. Those results suggest that the function of XXXL/R is located upstream of *daf-16* in the dauer regulatory pathway and that the function of *daf-9* in other cells is located downstream of or in parallel with *daf-16* in the pathway.

The XXXL/R-specific suppression by *daf-16* suggests that the DAF-2 insulin receptor signal transduction may play a tissue-specific role in these cells, which involves SDF-9 function and whose defect leads to dauer-like larvae formation. Experiments on *daf-2* mosaic animals (Apfeld and Kenyon, 1998) revealed that animals that are *daf-2(–)* in AB, ABa, ABar, ABp, or ABplaa cell lineages form dauer-like larvae (‘class II dauers’). Since XXXL and XXXR cells form in the ABplaa and ABarpa lineages, respectively, the result is in agreement with the idea that loss of *daf-2* gene activity in XXXL/R results in the formation of dauer-like larvae. It remains to be examined whether *daf-2* is expressed in XXXL/R and whether SDF-9 interacts with the DAF-2 tyrosine kinase, especially its tyrosine-phosphorylated form.

XXXL/R cells are involved in dauer regulation, probably through steroid hormonal signaling and/or steroid metabolism

We identified the *sdf-9*- and *daf-9*-expressing cells in the head as XXXL/R by mosaic experiments. These cells are known as embryonic hypodermal cells whose function at later stages is

unknown (Sulston et al., 1983). White et al. (White et al., 1986) included these cells in the list of neurons and their associated cells in the head (figure. 2 of White et al.). This study revealed a function of XXXL/R, namely, regulation of dauer formation. The most direct evidence for this function is that killing of these cells induces formation of dauer-like larvae in the wild-type background and that of dauer larvae in the *unc-31* background.

Gerisch et al. (Gerisch et al., 2001) and Jia et al. (Jia et al., 2002) also carried out laser microsurgery experiments on these cells. Although their results are partly different from ours, we think there is no major contradiction. Gerisch et al. (Gerisch et al., 2001) reported that ablation of *daf-9*-expressing head cells in 41 *daf-9(dh6)*; *Is*[*daf-9*::GFP] animals resulted in dauer-like larva formation in only four (10%) of them. The difference may be due to overexpression of *daf-9* by *Is*[*daf-9*::GFP] as well as other differences in experimental conditions. Jia et al. (Jia et al., 2002) found that none of the six N2; *Ex*[*daf-9p*::GFP] animals formed dauer-like larvae after killing of the *daf-9*-expressing head cells, but they argued that the timing of the surgery might be too late. They also killed these cells in *daf-9(m540)*; *Ex*[*daf-9p*::*daf-9* cDNA::GFP] animals, and all the four animals in which the surgery was successful formed dauer-like larvae.

XXXL/R cells seem to regulate dauer formation through the functions of *daf-9* and *sdf-9*, which are expressed in these cells and whose mutants form dauer-like larvae. The following results show that these functions are probably related to steroid hormonal signaling and/or steroid metabolism. First, *daf-9* encodes a cytochrome P450 of the CYP2 family, whose members are involved in steroid metabolism (Gerisch et al., 2001; Jia et al., 2002). Second, the *daf-12* gene, which acts downstream of *daf-9*, encodes a steroid hormone receptor (Antebi et al., 2000). Third, the Daf-c phenotypes of *daf-9*, *sdf-9* and *daf-12(daf-c)* mutations are enhanced by the deprivation of cholesterol (Gerisch et al., 2001; Jia et al., 2002; this study). Fourth, the expression of SDF-9 under the control of the *npc-1* promoter rescues the Daf-c phenotype of *unc-31(e169); sdf-9(ut187)*, where *npc-1* is a homolog of the Niemann-Pick type C disease gene, which plays a role in intracellular sterol transport (Hoekstra and van IJzendoorn, 2000; Ioannou, 2001).

In conclusion, we showed that SDF-9, a protein tyrosine phosphatase-like molecule, is involved in the regulation of dauer formation. Its function is closely related to that of DAF-9, a P450 molecule: it probably enhances the activity of DAF-9 or helps the execution of the DAF-9 function. Furthermore, SDF-9 is expressed in two head cells in which DAF-9 is expressed. We identified these cells as XXXL/R cells, which are known as embryonic hypodermal cells but whose function at later stages remains to be studied. Since this study on SDF-9 and former studies on DAF-9 suggested that the functions of these proteins are related to steroid metabolism or steroid hormonal signaling, XXXL/R cells seem to play a key role in the metabolism or function of a steroid hormone(s) that acts in dauer regulation.

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Supplemental Data for Ohkura et al. (2003)

Estimation of the numbers of extrachromosomal array losses in mosaic animals

We first enumerated all the theoretically possible cell lineages of the two RFP-expressing cells and two GFP-expressing cells, of which we can distinguish the left-hand cell and the right-hand cell (Fig. S1A). We also enumerated all the possible mosaic patterns (Table S1, left column), where the mosaic animals were identified by the presence of *sdf-9p*::RFP in only one of the two *sdf-9*-expressing cells (RL or RR). For all the combinations of the cell lineages and the mosaic patterns, we estimated the numbers of extrachromosomal array losses by the method shown in Fig. S1B, and listed them in Table S1.

The numbers of animals showing various mosaic patterns were obtained by experiments using five cell-specific GFP markers (Table S2, column "animals"). The cell lineages of GFP- and RFP-expressing cells were deduced from Fig. 6B for each pair of GFP marker cells and RFP candidate cells. Table S2 (top row) shows the cell lineages by the notation of Fig. S1a ((a) to (o)), on the assumption that RFP-expressing cells were XXXL/R. The numbers of extrachromosomal array losses for each combination of the cell lineage and the mosaic pattern were taken from Table S1 and shown in the "loss" column of Table S2. From this Table we calculated the percentages of animals having one, two and three losses among the 128 mosaic animals tested (Table S3). The percentages for other candidates of RFP-expressing cells were calculated in a similar way and also shown in Table S3.

Rescue data

The rescue of the *unc-31;sdf-9(u187)* Daf-c phenotype by *daf-9p::sdf-9* and that of the *sdf-9(ut163)* Daf-c phenotype by SDF-9::GFP are shown in Tables S4 and S5, respectively.

FIGURE LEGENDS

Fig. S1. Estimation of the number of extrachromosomal array losses for each cell lineage and

misaic pattern.

(A). All the theoretically possible cell lineages of two RFP-expressing cells and two GFP-expressing cells.

In the mosaic experiments of this paper, we examined the presence of RFP in two cells (RL and RR) and GFP in two cells (GL and GR) in each animal, where RL and GL are on the left side of the head and RR and GR on the right side. This figure shows all the theoretically possible cell lineages for the four cells. Only the topology of the cell lineages is relevant in this figure.

(B) Examples of estimating the number of extrachromosomal array losses for each combination of cell lineage and mosaic pattern.

This figure shows examples of the mosaic pattern in which RL, GL and GR have lost the extrachromosomal array but RR retains it (RL-, RR+, GL-, GR-). Such a mosaic pattern can be explained by one, two and three extrachromosomal array losses for the cell lineages (g), (f), and (e), respectively. Horizontal double-lines show the position where the extrachromosomal array is lost. The results for all the cases are shown in Table S1.

Table S1. Minimal number of extrachromosomal array losses required to explain each combination of cell lineage and mosaic pattern

mosaic pattern		cell lineage														
		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)	(n)	(o)
RL+, RR-	GL+, GR+	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RL+, RR-	GL+, GR-	2	1	2	2	2	2	2	2	2	2	2	1	1	2	2
RL+, RR-	GL-, GR+	2	2	1	2	2	2	2	2	2	1	1	2	2	2	2
RL+, RR-	GL-, GR-	2	2	2	3	3	3	3	3	3	2	1	2	1	2	1
RL-, RR+	GL+, GR+	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RL-, RR+	GL+, GR-	2	2	1	2	2	2	2	1	1	2	2	2	2	2	2
RL-, RR+	GL-, GR+	2	1	2	2	2	1	1	2	2	2	2	2	2	2	2
RL-, RR+	GL-, GR-	2	2	2	3	3	2	1	2	1	3	3	3	3	1	2

See Fig.S1A for the cell lineages (a) to (o), and Fig.S1B for the method by which the numbers of extrachomosomal array losses were estimated. See the legends of Fig.S1 for RL+, etc.

Table S2. Mimal number of extrachromosomal array losses on the assumption that RFP were expressed in XXXL/R.

mosaic pattern		GFP marker									
		gpa-4 ::GFP (g)		*ttx-3* ::GFP (g)		*gcy-8* ::GFP (c)		T23G5.5::GFP (b)		*egl-4.a* ::GFP (m)	
		animals	loss	animals	loss	animals	loss	animals	loss	animals	loss
RL+, RR-	GL+, GR+	16	1	17	1	5	1	6	1	8	1
RL+, RR-	GL+, GR-	1	2	1	2	1	2	6	1	0	1
RL+, RR-	GL-, GR+	0	2	1	2	3	1	0	2	0	2
RL+, RR-	GL-, GR-	0	3	0	3	1	2	0	2	3	1
RL-, RR+	GL+, GR+	2	1	4	1	7	1	3	1	16	1
RL-, RR+	GL+, GR-	1	2	0	2	3	1	0	2	0	2
RL-, RR+	GL-, GR+	2	1	1	1	1	2	13	1	0	2
RL-, RR+	GL-, GR-	3	1	2	1	1	2	0	2	0	3

(g), (c), (b) and (m) indicate the cell lineages in Fig. S1A.

"animals" means the number of animals having the mosaic pattern shown on the left, as determined by experiments.

"loss" is the minimal number of extrachromosomal array losses, as estimated from the cell lineage and the mosaic pattern (Table S1).

Of the 128 mosaic animals in this table, 120, 8, and 0 suffered extrachomosomal loss once, twice, and three times, respectively.

Table S3. Relative frequency of mosaic animals that suffered loss of the extrachromosomal array once, twice, and three times, as estimated for each pair of candidates of the RFP-expressing cells.

Candidates of RFP-expressing cells	Nr of losses		
	1 loss	2 losses	3 losses
XXXL/R	94% (120/128)	6% (8/128)	0% (0/128)
ILshL/R	65% (83/128)	31% (40/128)	4% (5/128)
CEPsoVL/R	65% (83/128)	31% (40/128)	4% (5/128)
OLQshVL/R, OLLshL/R	69% (88/128)	25% (32/128)	6% (8/128)
URAVL/R, URYVL/R, CEPshVL/R	80% (103/128)	19% (24/128)	1% (1/128)

Table S4. Rescue of the *unc-31;sdf-9(u187)* Daf-c phenotype by *daf-9p::sdf-9* [a]

	Dauer larva formation at 25˚C[c]	
Strain[b]	+Ex[d]	-Ex[e]
line 1	1.6%(62)	98.6%(70)
line 2	0%(71)	98.2%(55)
line 3	0%(41)	91.4%(58)

[a] Three independent transgenic lines were examined.

[b] Representative genotype, *unc-31(e169);sdf-9(ut163);Ex[daf-9p::sdf-9, gcy-10::GFP]*.

[c] The numbers in parentheses show the numbers of animals scored.

[d] Animals carrying the extrachromosomal array.

[e] Aanimals that had lost the extrachomosomal array.

Table S5. Rescue of the Daf-c phenotype of *sdf-9(ut163)* by SDF-9::GFP[a]

	Dauer-like larva formation at 25˚C[c]	
Strain[b]	+Ex[d]	-Ex[e]
line 1	4.0%(101)	94.7%(95)
line 2	0.7%(144)	90.7%(75)
line 3	0.8%(129)	84.8%(99)

[a] Three independent transgenic lines were examined. Animals were grown on NGM minus cholesterol plates.

[b] Representative genotype, *sdf-9(ut163);Ex[SDF-9::GFP(pKOG7), rol-6(su1006)]*.

[c] The numbers in parentheses show the numbers of animals scored.

[d] Rol animals, which carried the extrachomosomal array.

[e] Rol^+ animals, which had lost the extrachomosomal array.

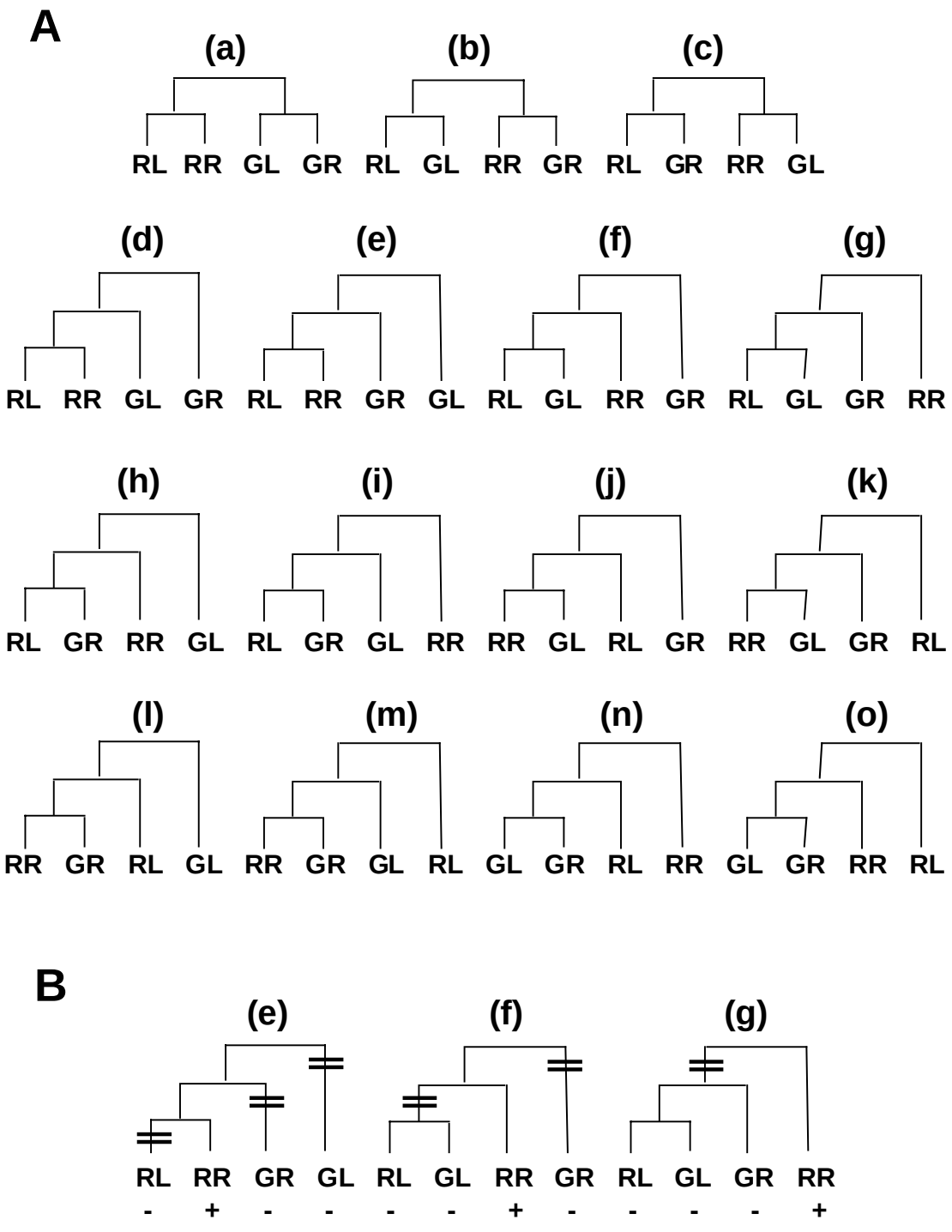

Fig. S1 Ohkura et al