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Note

Caenorhabditis elegans SDF-9 Enhances Insulin/Insulin-Like Signaling Through Interaction With DAF-2

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ABSTRACT

SDF-9 is a modulator of *Caenorhabditis elegans* insulin/IGF-1 signaling that may interact directly with the DAF-2 receptor. SDF-9 is a tyrosine phosphatase-like protein that, when mutated, enhances many partial loss-of-function mutants in the dauer pathway except for the temperature-sensitive mutant *daf-2(m41)*. We propose that SDF-9 stabilizes the active phosphorylated state of DAF-2 or acts as an adaptor protein to enhance insulin-like signaling.

IN an environment favorable for reproduction, *Caenorhabditis elegans* develops directly into an adult through four larval stages (L1–L4). Under conditions of overcrowding, limited food, or high temperature, larvae arrest development at the second molt to form dauer larvae (Cassada and Russell 1975). Dauer larvae can remain in diapause for months, essentially not aging, until conditions improve (Klass and Hirsh 1976). The signaling pathways that control the developmental switch have been revealed by studying two broad classes of mutants: those that block dauer formation (Daf-d, dauer formation defective) and those that result in constitutive dauer or dauer-like arrest (Daf-c), even when conditions are favorable (reviewed by Riddle and Albert 1997).

The *daf* genes encode elements of pathways conserved in humans, including insulin/IGF-1 (IIS), *t*ransforming *g*rowth *f*actor-β (TGF-β), *t*arget *o*f *r*apamycin, and guanylate cyclase/G-protein-mediated pathways (reviewed by Hafen 2004; Levy and Hill 2006). In addition to the Daf-c phenotype, mutants with reduced IIS signaling also display an adult life-span extension (Age) phenotype (Kenyon *et al.* 1993; Larsen *et al.* 1995).

Higher growth temperatures favor dauer formation, as observed in wild-type strains (*C. elegans* N2 and wild-type *C. briggsae*) exposed to exogenous dauer pheromone (Golden and Riddle 1984a; Jeong *et al.* 2005; Butcher *et al.* 2007) and in hypomorphic Daf-c mutants, which convey increased pheromone sensitivity (Golden and Riddle 1984b). The concentration of dauer pheromone indicates population density. Among the Daf-c mutants, null mutants of TGF-β pathway genes convey a temperature-sensitive (ts) phenotype, whereas severe *daf-2* or *age-1* mutants (IIS pathway) arrest at the dauer stage nonconditionally (Larsen *et al.* 1995; Ren *et al.* 1996). The IIS pathway is essential for larval maturation beyond the dauer stage, whereas the TGF-β pathway is essential only at higher temperatures as long as the IIS pathway is intact. Most Daf-c alleles are temperature sensitive not as a result of a thermolabile protein product but because temperature is an input into dauer formation (Golden and Riddle 1984a). One truly temperature-sensitive allele has been described, *daf-2(m41)*, which is wild type at 15°, but severe at 25° (Gems *et al.* 1998). This is unlike other *daf-2* alleles, which have partially penetrant Daf-c phenotypes at 15° that correlate with their severity at 20°, indicating that they are hypomorphic.

Forward mutagenesis screens have been useful, not only for understanding dauer biology, but also because the cloning of Daf-c and Daf-d genes has identified novel members of these conserved pathways (Georgi *et al.* 1990; Ren *et al.* 1996; Ogg *et al.* 1997; Patterson *et al.* 1997; Paradis and Ruvkun 1998; Da Graca *et al.* 2004). There are likely to be more genes involved that modulate or coordinate these pathways (Tewari *et al.* 2004). There is also value in creating new alleles for genes already known to be involved in the dauer pathways. Transposon insertion mutants allow isolation of knockout mutations or *in situ* knockin alterations to the gene (Plasterk and Groenen 1992; Barrett *et al.* 2004). In the *mut-2* (mutator) genetic background, mobilized

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transposons of the Tc family insert into or near genes preferentially at consensus sites (Collins *et al.* 1987). We used *mut-2* in a screen to identify novel Daf-c mutants and to obtain transposon insertion alleles for genes already known (Caldicott 1995).

Isolation and mapping of *m708*: One nonconditional Daf-c mutant from the *mut-2* screen failed to complement *daf-2(e1370)*. Upon subsequent backcrossing, it was determined to contain not only a ts allele of *daf-2, m637*, but also an independently segregating *daf-2* enhancer, *m708*. The *m708* homozygous single mutant exhibited only a weak egg laying phenotype and no evidence of Daf-c, Hid (Daf-c at 27°), neuronal dye filling, or Age phenotypes previously associated with Daf mutants (Malone and Thomas 1994; Larsen *et al.* 1995; Ailion and Thomas 2000).

Three-factor mapping placed *m708* on chromosome V to the right of *unc-51*. Polymerase chain reaction (PCR) amplification of six candidate genes using gene-specific primers revealed that one gene, *sdf-9* (synthetic dauer formation), had a 1.2-kb transposon insertion in exon 4. On the basis of dauer formation on NGM agar plates without cholesterol (Ohkura *et al.* 2003), *m708* failed to complement *sdf-9(ut163)*. The insert is contained between C515 and T516 of the coding sequence and is nine bases to the left of the lesion in *ut163* and nine bases to the right of the lesion in the *ut169* and *ut174* alleles (Ohkura *et al.* 2003). The *m708* open reading frame ends with an amber stop 24 codons into the insert. No *sdf-9* mRNAs were detected in either of two *sdf-9(m708)* RNA preparations, but we cannot eliminate the possibility that an altered gene product may be produced.

***Cemar1* transposition:** Unexpectedly, *sdf-9(m708)* did not contain a transposon normally mobilized by *mut-2* (*i.e.*, Tc1 or Tc5). Instead, it contained *Cemar1* (*C. elegans* Mariner 1), found in 66 copies in the haploid genome of the N2 strain, but not previously reported to be mobile (Witherspoon and Robertson 2003). *Cemar1* was originally identified by the repeat identifying program RECON (Bao and Eddy 2002) and is currently listed by WormBase as Ce000178 (release WS172; http://www.wormbase.org/).

The sequence of the inserted element differed from the consensus by an 11-bp deletion and a T > C mutation. The only copy of *Cemar1* with the corresponding sequence is located on chromosome V, 10 Mbp to the left of *sdf-9* in an intron of the gene D1054.5, indicating that the transposon inserted in *sdf-9(m708)* originated from this copy. The parental *mut-2* strain used for the mutant screen contained no transposon in *sdf-9*, whereas the original isolate of *sdf-9(m708)* still contained the parent transposon in D1054.5 as well as the insert in *sdf-9(m708)*. Most copies of *Cemar1* encode a functional transposase (Witherspoon and Robertson 2003), the expression of which has been observed in at least two expression studies (Kim *et al.* 2001; Murphy *et al.* 2003). We conclude that *Cemar1* was mobilized in the *mut-2* background, and other uncharacterized mutants isolated in this background may bear insertions of *Cemar1* rather than members of the Tc family.

Epistatic analysis of *sdf-9* and *daf-c* mutants: Since *sdf-9(m708)* enhanced *daf-2(m637)*, double mutants were created with other representative alleles of *daf-2* to determine if the enhancement is allele specific. As was the case with *daf-2(m637)*, the *daf-2(e1370); sdf-9(m708)* double mutant showed a much stronger Daf-c phenotype compared to *daf-2(e1370)* (Table 1). Double mutants between *sdf-9(m708)* and the strong ts *daf-2* alleles, *e979* (Table 1) and *m637* (data not shown), were nonconditional, forming only nonrecovering dauer larvae at 15°.

We also tested *daf-2(e1365)* and *daf-2(e1368)*, two alleles with very weak Daf-c phenotypes, and *daf-2(m41)*, a unique ts mutant exhibiting no Daf-c or Age phenotype at 15°, but a stronger Daf-c phenotype at 22.5° than other *daf-2* alleles that are Age at 15° (Gems *et al.* 1998). At 20°, 22°, and 23°, *m708* strongly enhanced both *e1365* and *e1368*. However, *m708* did not enhance *m41* at any temperature at which enhancement might be detected (Table 1).

To investigate possible *sdf-9* allele specificity in the interaction with *daf-2*, we made *sdf-9(ut163)* double mutants with *daf-2(m41)* and *daf-2(e1370)*. As was the case with *m708*, *ut163* enhanced *daf-2(e1370)* dauer formation but not *daf-2(m41)* (Table 1). The *ut163* allele appears to be ts because it has a very weak phenotype at 15° [no enhancement of *daf-2(e1370)*], a moderate phenotype at 20° (weaker enhancement than *sdf-9(m708)*), and a stronger Daf-c phenotype than *m708* at 25° (Table 1). Ohkura *et al.* (2003) reported that the *ut163* Daf-c phenotype was the second strongest among the tested *sdf-9* alleles at 25° but the weakest allele at 20° on NGM plates lacking cholesterol, also indicating that the *ut163* protein may be thermolabile.

Since *sdf-9* enhanced all of the *daf-2* alleles tested except for *m41*, it could be judged to fall into a parallel dauer formation pathway. *sdf-9(m708)* also enhanced *daf-7(e1372)* and *daf-8(m85)*, which encode components of the TGF-β pathway (Table 2). In fact, double mutants between *sdf-9(m708)* and the type I and type II TGF-β receptors, *daf-1(m40)* and *daf-4(e1364)*, respectively, constitutively formed dauer larvae that could not recover at 15° (data not shown). Furthermore, the Daf-c phenotype of *daf-11*, encoding a transmembrane guanylate cyclase (Birnby *et al.* 2000), was enhanced by *sdf-9*. *daf-11(m47) sdf-9(m708)* exhibited strong dauer formation at 15° (Table 2), with many dauer larvae unable to resume development. Taken together, it appears that SDF-9 may work in parallel to the TGF-β and guanylate cyclase pathways.

sdf-9 not only enhanced the phenotypes of all tested Daf-c mutants except for *daf-2(m41)*, but also proved to be hypersensitive to dauer pheromone, as are the Daf-c mutants (Golden and Riddle 1984b). *sdf-9(m708)*

TABLE 1

Interaction between *sdf-9* and *daf-2*

	15°		20°		22°		23°		25°	
Genotype	Daf-c	*N*	Daf-c	*N*	Daf-c	*N*	Daf-c	*N*	Daf-c	*N*
daf-2(m41)	0	129	10 ± 5	365	33 ± 3	393	100	>500[a]	100	131
sdf-9(m708)	0	300	0	370	0	280	—	—	0	140
m41; m708	0	72	5 ± 6	133	30 ± 5	332	100	>500[a]	100	96
sdf-9(ut163)	0	258	0	667	0	321	—	—	17 ± 2	110
m41; ut163	0	340	4 ± 2	339	31 ± 6	395	—	—	100	126
daf-2(e1368)	—	—	1 ± 1	195	6 ± 1	388	8 ± 3	250	—	—
e1368; m708	—	—	60 ± 17	424	89 ± 6	340	100	220	—	—
daf-2(e1365)	—	—	1 ± .3	381	4 ± 2	544	16 ± 8	1277	—	—
e1365; m708	—	—	14 ± 4	280	53 ± 15	131	100	1120	—	—
daf-2(e1370)	0	683	3 ± .4	1131	—	—	—	—	—	—
e1370; m708	92 ± 3	628	100	>500[a]	—	—	—	—	—	—
e1370; ut163	0	766	50 ± 4	959	—	—	—	—	—	—
daf-2(e979)	10[b]	30	—	—	—	—	—	—	—	—
e979; m708	100[b]	30	—	—	—	—	—	—	—	—

Values are percentage of dauer larvae ± standard error, including dauer and dauer-like larvae counted on the first day of adulthood for non-dauer siblings. Populations were synchronized by hatching alkaline hypochlorite-treated embryos in M9 buffer at room temperature for 24 hr and then transferring them to NGM plates with *Escherichia coli* OP50. *N*, population size. Alleles are listed in order of severity except *m41*, which is listed first because of its unique phenotype. Strains were genotyped as follows: PCR for *sdf-9(m708)*, sequencing for *daf-2(m41)* and *sdf-9(ut163)* and by phenotype for all other *daf-2* alleles.

[a] Based on visual inspection of multiple samples (>500 animals) with no larvae growing past dauer.

[b] Includes genotyped progeny from one *daf-2(e979)/daf-2(e979); sdf-9(m708)/+*, including 12 dauers, 35 nonrecovering dauers (allowed to recover at 15° for 3 days after cold shock at 4° overnight) and 53 adults (χ^2 *P*-value <0.001).

formed far more dauer larvae than N2 at all temperatures and concentrations of pheromone tested (Figure 1). We then subjected *sdf-9(m708)*; *daf-d* double mutants from the IIS (*daf-16*) and TGF-β (*daf-3*) pathways to dauer pheromone at 2.5 μl/plate to see which pathway mediates the hypersensitivity. *daf-16(m26); sdf-9(m708)* failed to form dauer larvae in response to pheromone ($N = 114$), but *sdf-9(m708); daf-3(e1376)* formed only slightly fewer dauer larvae (76 ± 6%, $N = 119$) than *sdf-9(m708)* alone (100%, $N = 124$). Neither the *daf-3(e1376)* nor the *daf-16(m26)* single mutant formed any dauer larvae ($N = 116$ and 102, respectively). This suggests that SDF-9 modulates the IIS pathway, since *daf-16*(+) is required for *sdf-9* to manifest its effect, whereas *daf-3*(+) is not required.

daf-16 suppression of *sdf-9* hypersensitivity to dauer pheromone is dominant. We crossed *daf-16(m26)/+; sdf-9(m708)/+* males to *sdf-9(m708); dpy-3(e27)* double mutants and exposed the progeny to 2.5 μl of dauer pheromone. If *daf-16* were a recessive suppressor, we would expect 50% dauer larvae among the hermaphrodite progeny, but we observed only 27 ± 7% ($N = 64$) for the line used in the pheromone assay and 21 ± 6% ($N = 63$) for another isolate of the same genotype. This is compared to 100% dauer larvae in the control *sdf-9(m708)* strain ($N = 120$) and 0% for N2 ($N = 114$). Since a 50% reduction in *daf-16*(+) gene dosage suppresses dauer formation, high levels of DAF-16 activity must be required for expression of the *sdf-9* mutant phenotype.

Enhancement of *daf-2* by *sdf-9(m708)* was also semidominant. As shown in Table 1, 100% of the progeny of *daf-2(e1368); sdf-9(m708)* formed dauer larvae compared to 8% dauer larvae for *daf-2(e1368)* at 23°. If *sdf-9(m708)* were recessive, the expected number of dauer larvae segregated from *daf-2(e1368); sdf-9(m708)/+* heterozygotes would be 31% [= 25% *sdf-9*(−/−) + 8% × 75% *sdf-9*(+/− and +/+) = 31%], but we observed 41 ± 3% ($N = 624$). We conclude that *sdf-9(m708)* is a semidominant enhancer of *daf-2* (*P*-value from $\chi^2 = 4.5 \times 10^{-4}$). A 50% reduction in *sdf*-9(+) gene dosage enhances the

TABLE 2

Effect of *sdf-9* on mutants in other dauer pathways

	15°	
Genotype	Daf-c[a]	*N*
daf-7(e1372)	4 ± 2	465
e1372; m708	64 ± 3	177
daf-8(m85)	1 ± 0	481
m85; m708	63 ± 6	175[b]
daf-11(m47)	4 ± 2	330
m47 m708	85 ± 6	106

[a] Percentage of constitutive dauer formation ± standard error, with populations scored as in Table 1. Genotype was confirmed by phenotype for *daf-7(e1372)*, *daf-8(m85)*, and *daf-11(m47)* and by PCR for *sdf-9(m708)*.

[b] Approximately one-quarter of the animals in this sample were multivulva, small, embryonic lethal, grew slowly, or had other morphological defects. These were not included in the counts.

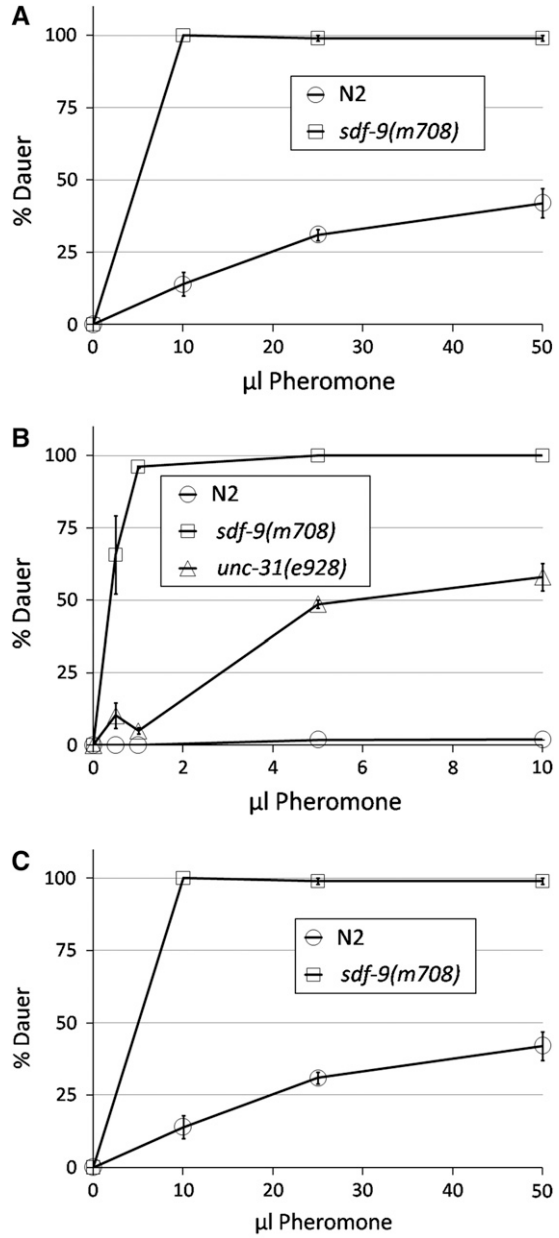

Figure 1.—Response of *sdf-9(m708)* to dauer pheromone. The pheromone extract and plates were made as previously described (Golden and Riddle 1984b) and used in the amounts given. (A and B) Each data point ± standard error represents results from two or three plates, each started with ~40 eggs laid *in situ* by three gravid adults, which were subsequently removed. Plates were scored for dauer formation on the first day of adulthood. At 25.5°, *sdf-9(m708)* showed increased sensitivity to pheromone for repeated experiments even when compared to *unc-31(e928)*, previously reported to be sensitive to pheromone at 25.4° (Ailion and Thomas 2000). (C) Using the same method, hypersensitivity to pheromone was also observed at 20°.

phenotype of weak *daf-2* mutants. Both SDF-9 and DAF-16 are points of fine tuning for insulin-like signaling.

To test whether *sdf-9* mutants may be long lived, as are the Daf-c mutants in the IIS pathway (Kenyon *et al.* 1993; Larsen *et al.* 1995), we compared the life spans of

TABLE 3

Maturation time

Genotype	20°	22°
N2	64	56
daf-2(m41)	104	96
sdf-9(m708)	64	56
sdf-9(ut163)	64	56
m41; m708	104	88
m41; ut163	64	80

Populations of 50–120 animals/plate (8–10 plates/genotype) were synchronized as in Table 1 prior to transfer to NGM agar plates with *E. coli* at 20° and 22°. They were observed every 8 hr until all 8–10 plates for each sample had eggs present. Within each strain there was no variation observed among plates.

sdf-9(m708) with N2 and *daf-2(e1370); sdf-9(m708)* with *daf-2(e1370)*. In agreement with the previous results for *sdf-9* (Ohkura *et al.* 2003; Hu *et al.* 2006), we saw no increase in the life span of *sdf-9(m708)* relative to N2. Similarly, *sdf-9(m708)* had no effect on the life span of *daf-2* (data not shown), despite enhancing its Daf-c phenotype. SDF-9 may function to enhance IIS primarily during larval development, and not during adulthood. By contrast, treatment of wild-type adults with *daf-2* RNA interference is sufficient to increase longevity (Dillin *et al.* 2002).

Slow maturation of Daf-c mutants to the adult stage at intermediate temperatures results from entry into an L2d-like state (the L2d is the pre-dauer L2 larva) with a delayed second molt (Swanson and Riddle 1981). *ut163* fully suppressed the slow growth of *daf-2(m41)* at 20°, whereas *m708* had no effect (Table 3). At 22°, both alleles partially suppressed the slow-growth phenotype. Suppression of the L2d delay would suggest a gain of SDF-9 function at the intermediate temperatures, but this is not supported by the dauer formation data (Table 1), which show no obvious suppression of the Daf-c phenotype at 20° or 22°. Instead, suppression of the slow-growth phenotype suggests that SDF-9 may interact with other pathways required for growth. For example, sterol deprivation has already been shown to enhance the weak Daf-c phenotype of *sdf-9* (Ohkura *et al.* 2003).

Model for SDF-9 function: We isolated an allele of *sdf-9* as an enhancer of *daf-2*. This gene was previously detected as an enhancer of *akt-1* (Hu *et al.* 2006) and *unc-31* (Ohkura *et al.* 2003). We found that *sdf-9* enhanced all Daf-c mutants tested, except *daf-2(m41)*. We propose that the allele specificity of interaction with *daf-2* and the requirement for *daf-16*(+), not *daf-3*(+), for SDF-9 function demonstrate genetically that SDF-9 modulates the IIS pathway, at least in larvae. These data support previous interpretations of SDF-9 function (Ohkura *et al.* 2003; Hu *et al.* 2006), which were based on its identity as a tyrosine-phosphatase-like protein

lacking the necessary catalytic cysteine residue and membrane-bound subcellular localization. These authors suggested that SDF-9 might act along with EAK-6 (another likely inactive tyrosine phosphatase that enhances the *akt-1* mutant phenotype) to bind the DAF-2-activating phosphotyrosine, but they did not test for genetic or molecular interaction with *daf-2*.

Tyrosine kinase receptors like DAF-2 function via ligand binding, dimerization, activation by trans-autophosphorylation, phosphorylation of target proteins, and deactivation by a tyrosine phosphatase (reviewed by Romano 2003). It is possible that SDF-9 enhances IIS signaling by protecting phosphorylated DAF-2 from inactivation by a tyrosine phosphatase, or it may act as an adaptor protein to enhance binding of a DAF-2 target to the DAF-2 kinase. Hypomorphic *daf-2* alleles would be sensitive to loss of SDF-9 as long as DAF-2 is able to trans-autophosphorylate.

The *daf-2(m41)* mutation is a G-to-A substitution that changes a glycine to glutamic acid at position 383 (Yu and Larsen 2001). The glycine is part of a Caenorhabditis conserved glycine–proline turn motif adjacent to a conserved cysteine in the cysteine-rich region in the extracellular domain. This structural change may disrupt a disulfide bond formed by the conserved cysteine and could make the overall structure of the *daf-2(m41)* gene product unstable or unable to dimerize at higher temperatures. Mutations in a similar domain of the EGFR protein cause an inability to dimerize, preventing trans-autophosphorylation (Macdonald *et al.* 2006). Lack of trans-autophosphorylation would render SDF-9 unable to modify *m41* activity, so loss of SDF-9 function would not affect the *m41* phenotype. Alternatively, thermolability of the *daf-2(m41)* protein may prevent binding of SDF-9 to DAF-2, also resulting in no enhancement of *daf-2(m41)* by *sdf-9(m708)*. We propose that all the hypomorphic *daf-2* alleles that are enhanced by *sdf-9(m708)* and *sdf-9(ut163)* trans-autophosphorylate at some level.

Formally, SDF-9 could bind to a phosphorylated target of DAF-2, rather than to DAF-2 itself. However, one would not expect *daf-2* allele specificity in that case, since the enhancement would be affected only by the phosphorylation state of the particular DAF-2 target. This explanation seems far less likely than our posited lack of *m41* protein dimerization.

STYX family proteins have tyrosine phosphatase domains that are phosphatase inactive (Wishart and Dixon 1998). They have been shown to bind proteins with phosphorylated serine, threonine, or tyrosine to act as adaptor proteins or to protect the phosphorylated tyrosine. Co-incubation of the mammalian STYX protein Sbf1 with SUV39H1, a mammalian ortholog of Drosophila Su(var)3-9 (suppressor of variegation), was shown to stabilize the phosphorylated state of SUV39H1, whereas engineering Sbf1 to restore catalytic phosphatase activity eliminated such stabilization (Firestein *et al.* 2000). In *C. elegans,* the STYX protein IDA-1 enhances *daf-2(e1370)*, apparently by functioning in several neurons likely to regulate insulin secretion (Cai *et al.* 2004).

On the basis of the genetic evidence provided by the *daf-2* allele specificity of interactions with *sdf-9* and the requirement for *daf-16(+)* to exhibit the *sdf-9* pheromone sensitivity, we propose that SDF-9 functions in the IIS pathway and binds to DAF-2 at one or more of the activating phosphotyrosines to enhance DAF-2 signaling during larval development. Loss of SDF-9 function reduces insulin-like signaling to enhance the phenotype of Daf-c mutants, even those in parallel pathways. If this interaction is conserved in other species, such phosphatase-like proteins could potentially be useful targets of novel therapies for diseases such as type 2 diabetes in humans.

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