## MATERIALS AND METHODS

### Strains

The wild-type strain used was *C. elegans* variety Bristol strain N2. Mutant strains were obtained from the National Bioresource Project (Japan), or the *Caenorhabditis* Genetics Center. Alleles used were *srbc-64(tm1946), srbc-66(tm2943)*, *gpa-2*(*pk16*), *gpa-3*(*pk35*), *gpa-14*(*pk342*), and *gpa-15*(*pk477*). The *srbc-64(tm1963)* strain contains an unlinked and complex genomic rearrangement that causes lethality and was not analyzed further. *srbc-64(tm1946)* and *srbc-66(tm2943)* strains were outcrossed at least three times prior to analysis. Strains carrying stably integrated transgenes were: *str-3*p*::gfp* (*kyIs128*), *daf-7*p*::gfp* (*mIs6*), and *sra-9*p::mCaspaseI (*qrIs1* and *2*: gift of Dr. R. Shingai). Double mutant strains were constructed using standard methods.

### Identification of SRBC-64 and SRBC-66 chemoreceptors

We carried out a genetic screen to identify mutants in which pheromone-mediated downregulation of *str-3*p*::gfp* expression was abolished. Animals carrying stably integrated *str-3*p*::gfp* fusion genes were mutagenized, and their F2 progeny grown in the presence of 3 μM crude pheromone extract were examined to identify animals retaining *str-3*p*::gfp* expression. Screening of ~40,000 haploid genomes yielded 84 mutants. Of these, 57 mutants exhibited defects in their ability to take up lipophilic dyes (*1, 2*), suggesting that their defects in pheromone-mediated gene expression arose due to morphological defects in neuronal sensory endings (*1*). Of the remaining 27 mutants, one mutation (*oy80*) genetically mapped to the genomic region encoding SRBC-64, and overexpression of *srbc-64* genomic sequences partially rescued the mutant phenotype.

Although *oy80* was subsequently found to not be an *srbc-64* allele, *srbc-64* null alleles (obtained from S. Mitani, National Bioresource Project, Japan) exhibited strong pheromone-mediated gene regulatory defects, and were investigated further. SRBC-66 was predicted to be the most closely related chemoreceptor to SRBC-64 in a phylogenetic analysis (*3*), and was thus also investigated.

**Dauer formation assays**

Five well-fed and growth-synchronized adult animals were allowed to lay eggs at 25°C for 4-5 hours on a 3.5-cm assay plate. Adult animals were then removed, and the plates containing approximately 50-100 eggs each were placed at 25°C for 68-72 hr. Dauer and nondauer animals were identified by visual inspection, and confirmed by selecting for dauer animals that survived 1% SDS treatment for 45 min. Assay plates were made with Noble agar (BD Biosciences) lacking peptone, and seeded with 160 μg heat-killed OP50 bacteria per plate. 6 μl ethanol, or ascaroside diluted to the appropriate concentration in ethanol, were added to each plate prior to adding agar. For each experiment, all strains were assayed in parallel in at least four independent experiments.

**Gene expression analyses**

Animals were grown as above, except that eggs were placed at 25°C until animals were at the adult stage. Assay plates were prepared as above, except that the agar contained peptone, and plates were seeded with live OP50 bacteria. These conditions precluded dauer formation. To quantify *str-3*p*::gfp* and *daf-7*p*::gfp* expression levels in the ASI neurons, images were captured with a Zeiss Axioplan microscope using a 40X (1.30 NA)

objective and a CCD camera (Hamamatsu) using a fixed exposure time. Fluorescence levels were quantified using OpenLab 4.0 software (Improvision).

**Real-time PCR**

Fifty well-fed growth-synchronized wild-type adult animals were allowed to lay eggs for 10 hours at 25°C on 10-cm plates containing crude pheromone and seeded with live OP50. Adult animals were then removed, and growth was allowed to proceed until animals were at the late L4 larval or young adult stage. Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were derived using oligo-d(T) primers, and used as template for quantitative real-time PCR performed with a Corbett Research Rotor-Gene 3000 real-time cycler, and primers specific for *str-1* and *str-3* coding sequences.

**Molecular Biology**

Rescuing constructs were generated by fusing *srg-8*, *srbc-64*, *srbc-66* or *srbc-65* regulatory sequences to *srbc-64*, *srbc-65*, or *srbc-66* cDNAs. For genomic rescue, ~4 kb of *srbc-64* genomic sequences containing ~1.8 kb of upstream regulatory sequences were amplified and directly injected into mutant animals. *srbc-64*p*::gfp* or *srbc-66*p*::gfp* expression constructs were generated by fusing ~1.8 kb of *srbc-64* or ~2.1 kb of *srbc-66* upstream regulatory sequences to *gfp* coding sequences in a *C. elegans* expression vector (gift of A. Fire). GFP-tagged full-length *srbc-64*/*srbc-66* constructs were generated using the PCR fusion method, and inserted into a *C. elegans* cloning vector (gift of M. Colosimo). *srbc-66*p::*gpa-3*[Q205L] or *srbc-65*p::*gpa-3*[Q205L] was generated by fusing

*srbc-66* or *srbc-65* regulatory sequences to a *gpa-3* cDNA containing the Q205L mutation, respectively. The Q205L mutation was generated using the QuikChange site-directed mutagenesis kit (Stratagene). Each plasmid was injected at 50 ng/μl with *unc-122*p*::dsRed* or *flp-8*p*::gfp* as the coinjection marker.

**Confocal microscopy**

Confocal images were acquired with a Leica confocal microscope equipped with 63X/1.4 NA objectives and Leica imaging software.

**Heterologous expression of SRBC-64/66**

Full-length cDNAs encoding SRBC-64 and SRBC-66 were cloned into the pME18S vector (*4*). Chemoreceptors were transiently transfected into HEK293 cells using Lipfectamine™ reagent (Invitrogen). Cells transfected with SRBC-64 and/or SRBC-66, mOR-EG (*5*), or a mock vector were incubated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min. The cells were then exposed to the indicated concentration of ligand solution together with 5 μM of forskolin for 15 min. cAMP levels were quantified using an enzyme-linked immunoassay (ELISA) kit following the manufacturer's directions (Applied Biosystems).

**Calcium Imaging**

Calcium imaging was performed in a custom-fabricated microfluidic device (Center for Nanoscale Systems, Harvard University), essentially as described (*6, 7*). Imaged animals were transgenic for an extrachromosomal array carrying *sra-9*p::*G-CaMP2.2b* fusion

genes (gift of C. Bargmann; (*7*)). G-CaMP fluorescence was imaged using a Olympus BX52WI microscope with a 40X objective and a CCD camera (Hamamatsu). Image processing and analyses were carried out using OpenLab 4.0 software (Improvision), ImageJ (NIH), and custom MATLAB (The Mathworks) scripts.

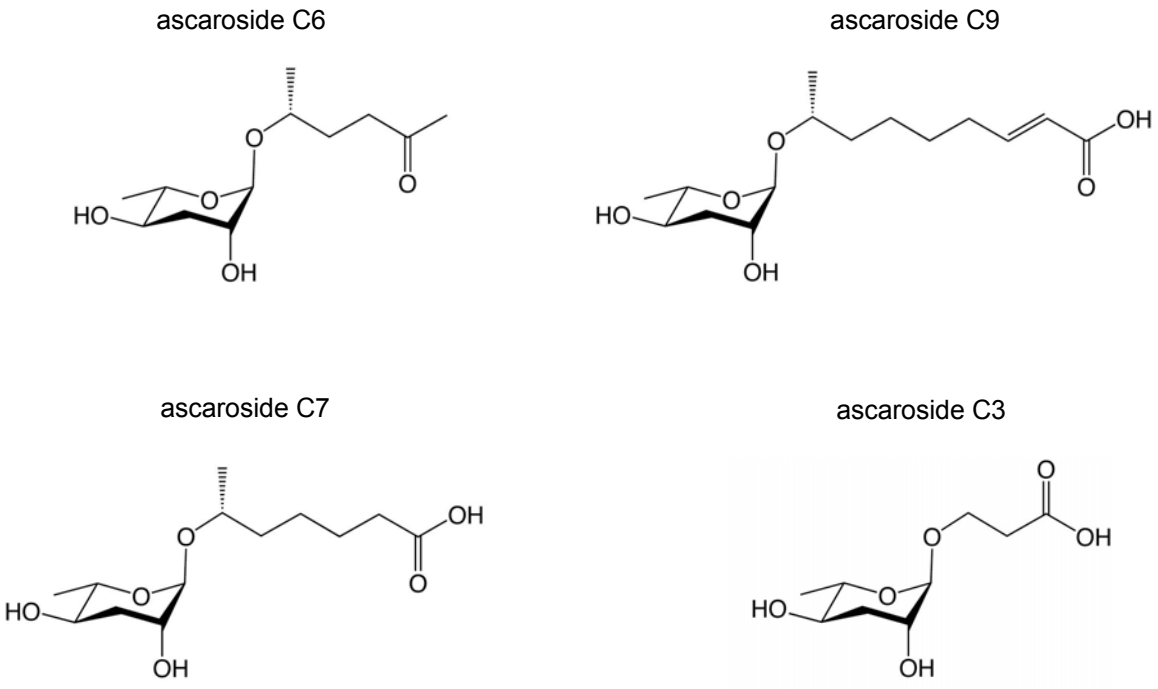


**Fig. s1.** Chemical structures of examined ascarosides. Ascarosides are referred to by the number of carbons in their side chains. From (*8, 9*).

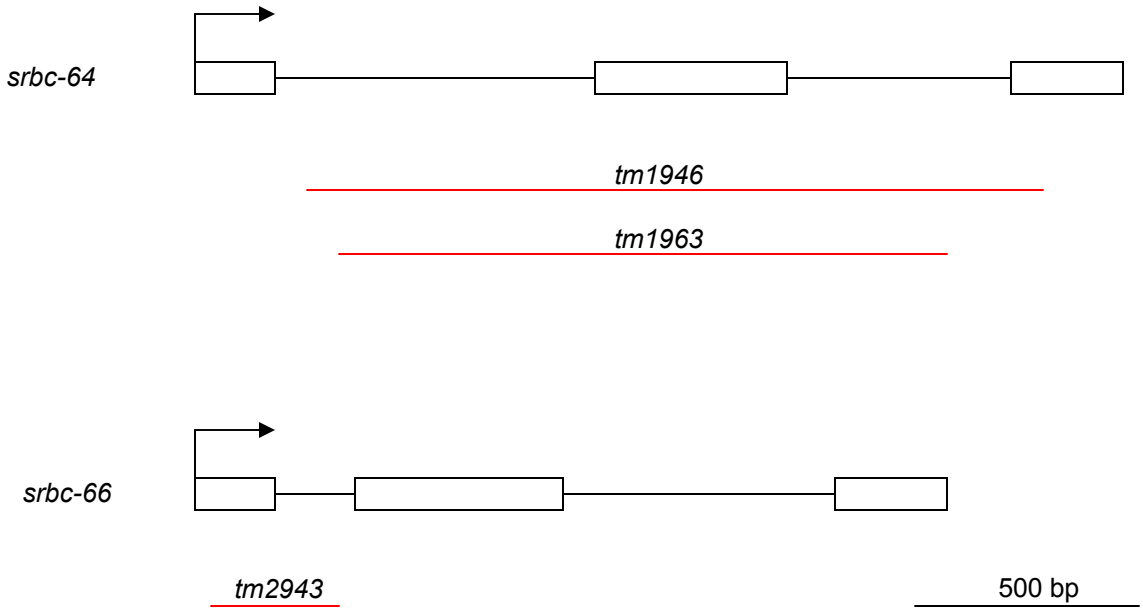


**Fig. s2.** Genomic structures of *srbc-64* and *-66*. Exons and introns are indicated by boxes and lines, respectively. The extent of the deletions in each mutant allele is indicated by a red line and was confirmed by sequencing.

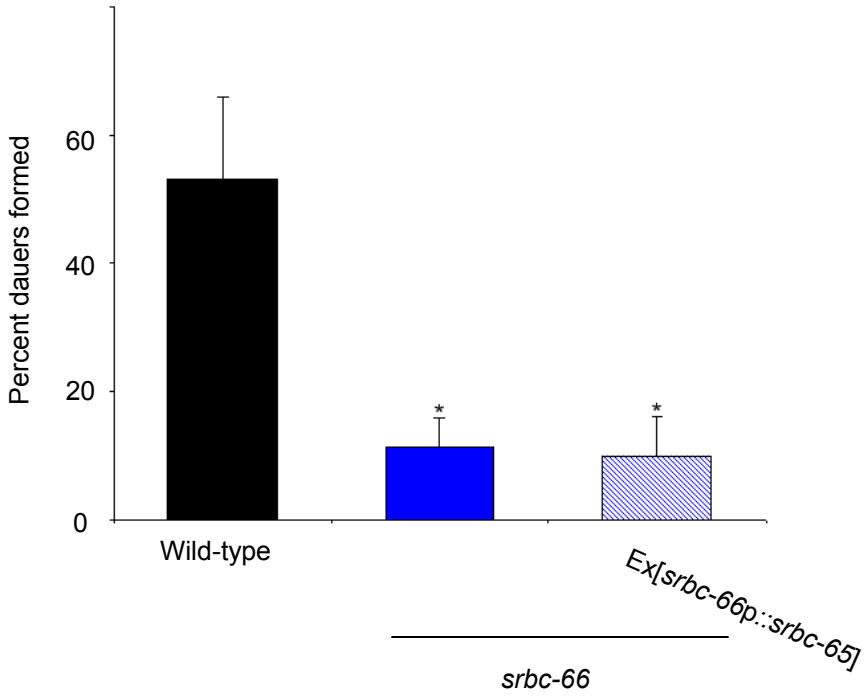


**Fig. s3.** Expression of the closely related *srbc-65* gene fails to rescue the *srbc-66* dauer formation defects. An *srbc-65* cDNA was expressed in the ASK neurons of *srbc-66* mutants under the *srbc-66* promoter. Dauer formation was assayed upon growth in 6 μM C6. All strains were assayed in parallel. Shown is the average of 4 independent experiments with 50-100 animals each. Error bars are the s.e.m. * indicates values that are different from that of wild-type at $P < 0.01$.

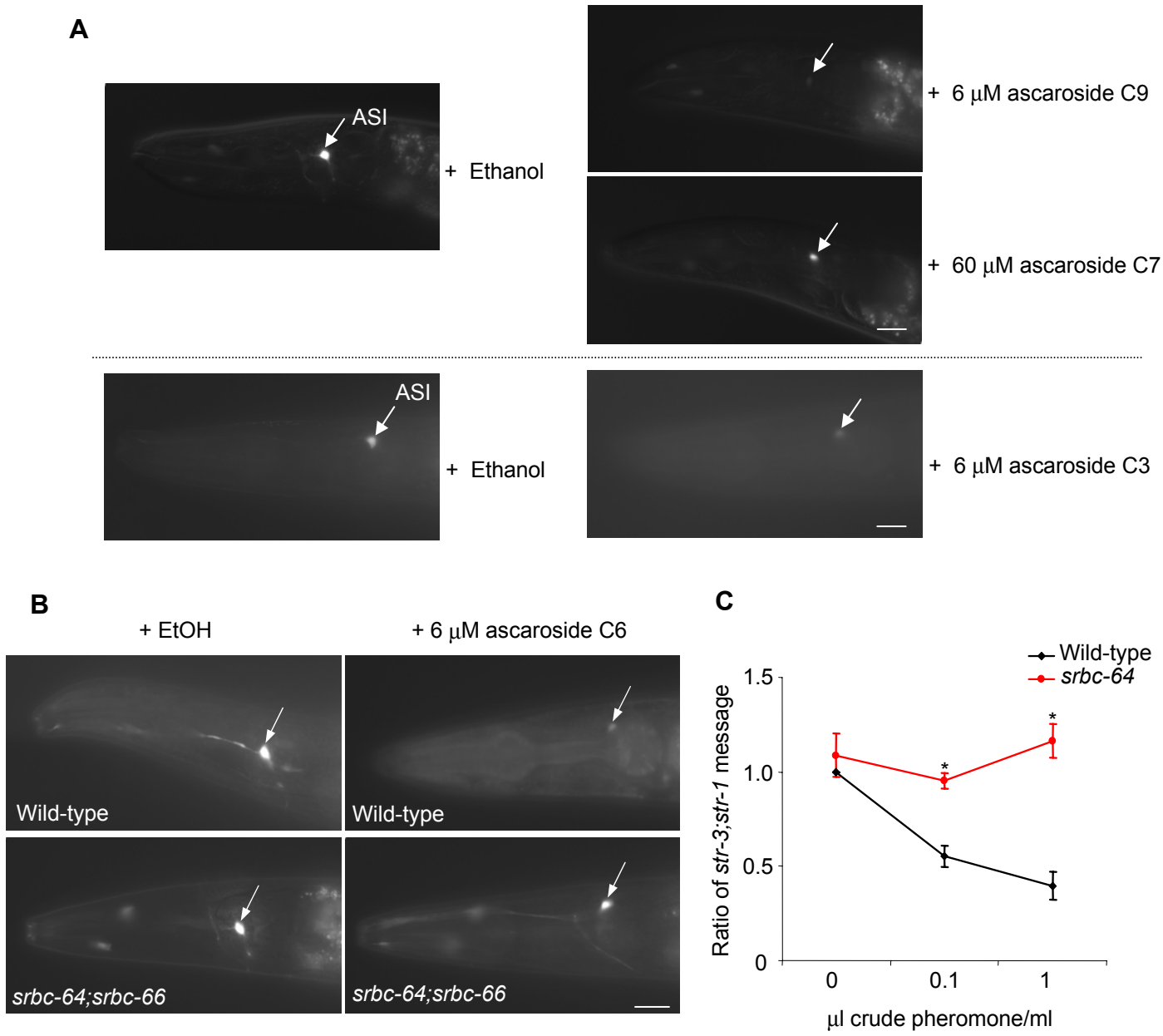


**Fig. s4.** *srbc-64* and *srbc-66* mediate pheromone-regulated repression of gene expression in the ASI neurons. **(A)** Shown is an ASI neuron expressing *daf-7*p*::gfp* in wild-type animals grown in the absence of exogenously added pheromone, or in the presence of C9, C7 and C3. Lateral view; scale – 10 µm. **(B)** Expression of *daf-7*p*::gfp* in wild-type and *srbc-64; srbc-66* mutant adult animals grown in the presence of 6 µM C6. Lateral view; scale – 10 µm. Adult animals were examined using the same exposure time. **(C)** Shown are the ratios of endogenous *str-3* message relative to *str-1* message in wild-type and *srbc-64* animals. *str-1* is expressed in the AWB neurons, and expression is unaffected upon addition of pheromone (*10*). The mean of the ratios of 4 independent RT-PCR experiments is shown. * indicates values that are different at $P<0.01$ from corresponding values of wild-type animals.

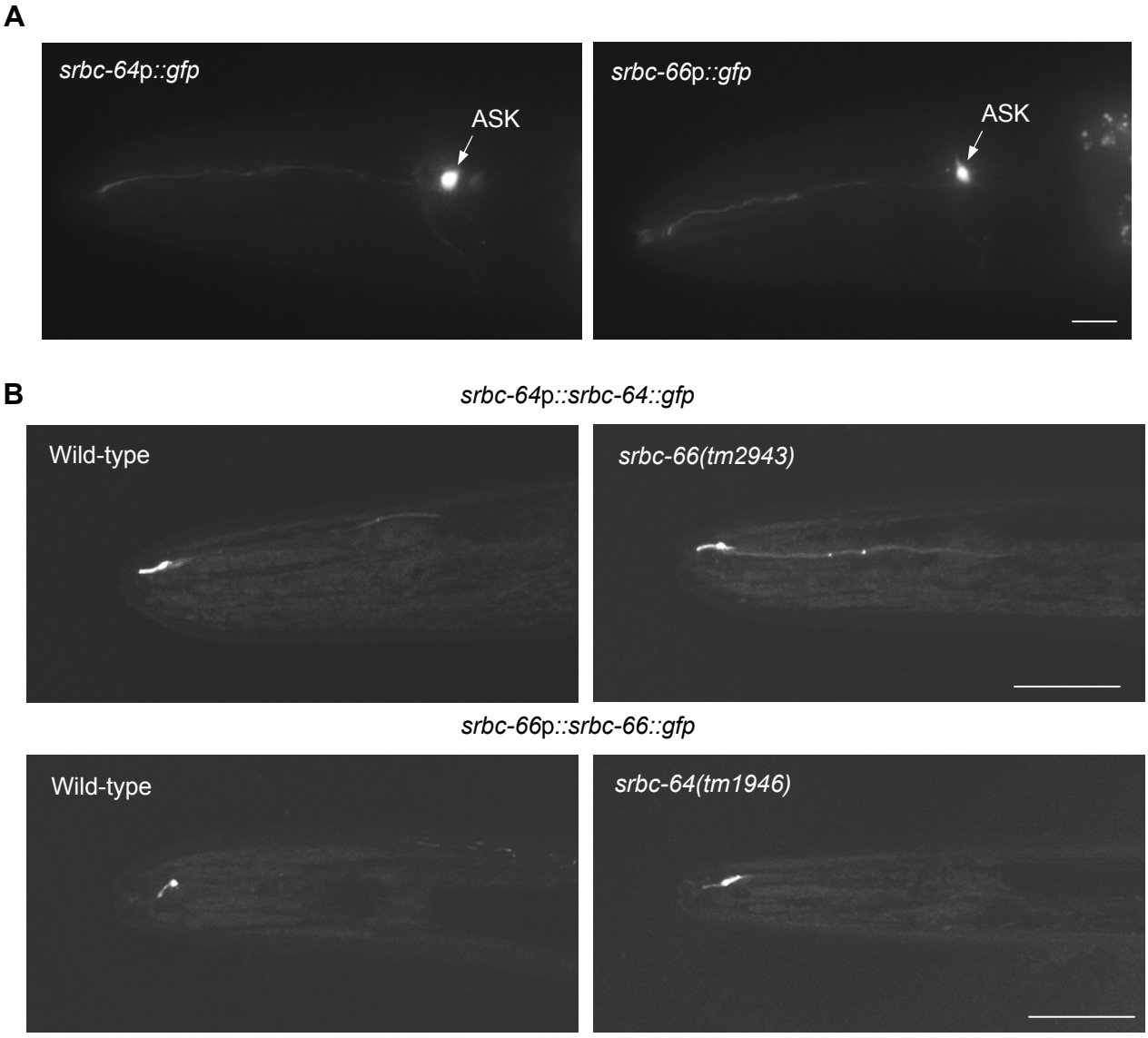


**Fig. s5.** *srbc-64* and *srbc-66* are expressed in the ASK neurons, and expression and localization of one chemoreceptor is unaffected in the absence of the other. **(A)** Transcriptional fusions of the upstream regulatory regions of *srbc-64* and *-66* to *gfp* drive expression in the ASK neurons (arrows). Adult animals were examined. **(B)** Shown is the localization of full-length GFP-tagged SRBC-64 and -66 proteins in the indicated genetic backgrounds. Adult animals were examined using the same exposure time on a confocal microscope. Lateral view; scale – 10 μm.

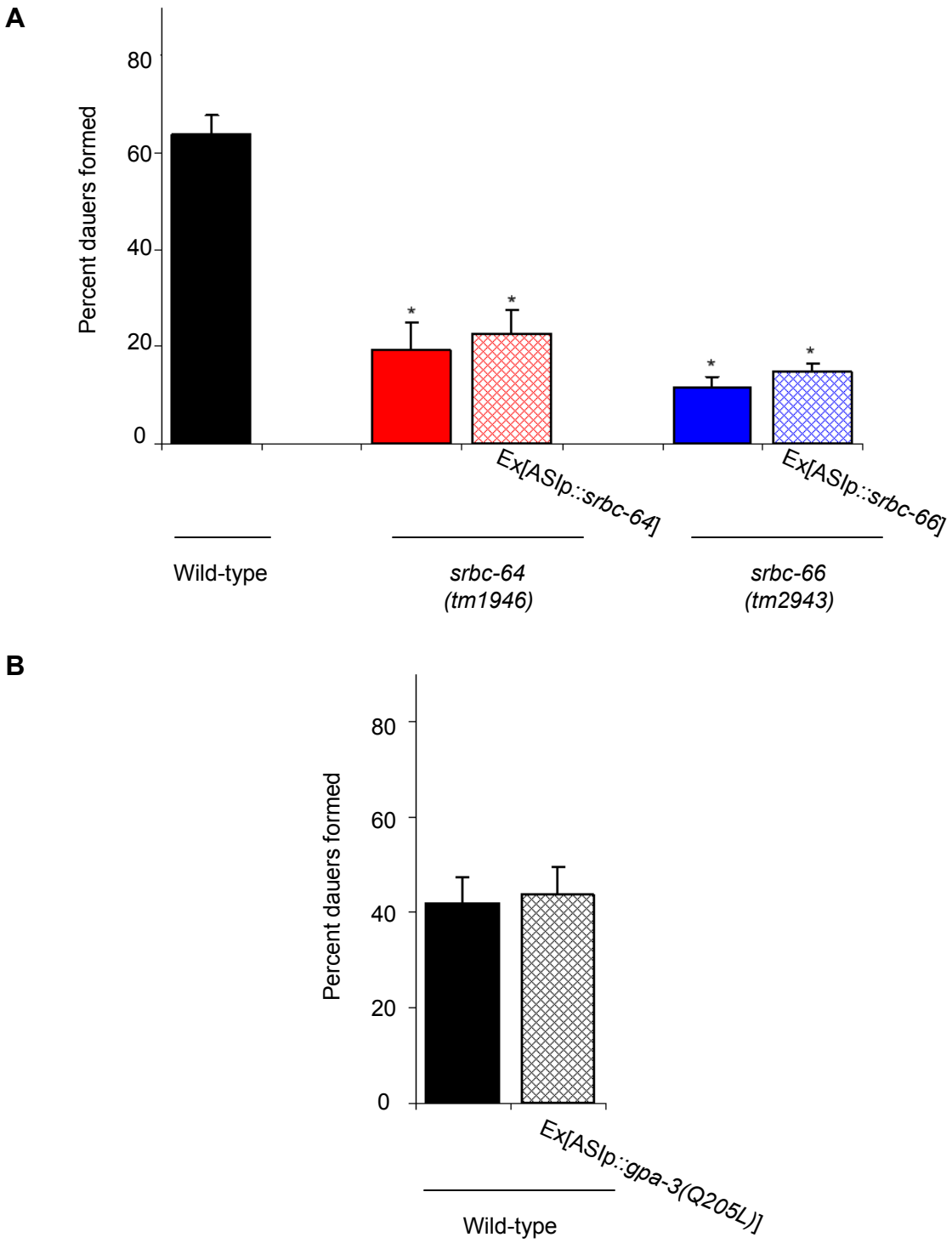


**Fig. s6.** SRBC-64 and SRBC-66 do not act in the ASI neurons. **(A-B)** Percent dauers formed when animals of the indicated genotypes were grown in the presence of 600 nM C6 **(A)** and 60 nM C6 **(B)**. *srbc-64, srbc-66* and *gpa-3(Q205L)* were expressed under the *srbc-65* promoter which drives expression exclusively in the ASI neurons. Shown are the averages from at least 4 independent experiments with 50-100 animals each. * indicates values that are differerent at P<0.01 from corresponding values of wild-type animals.

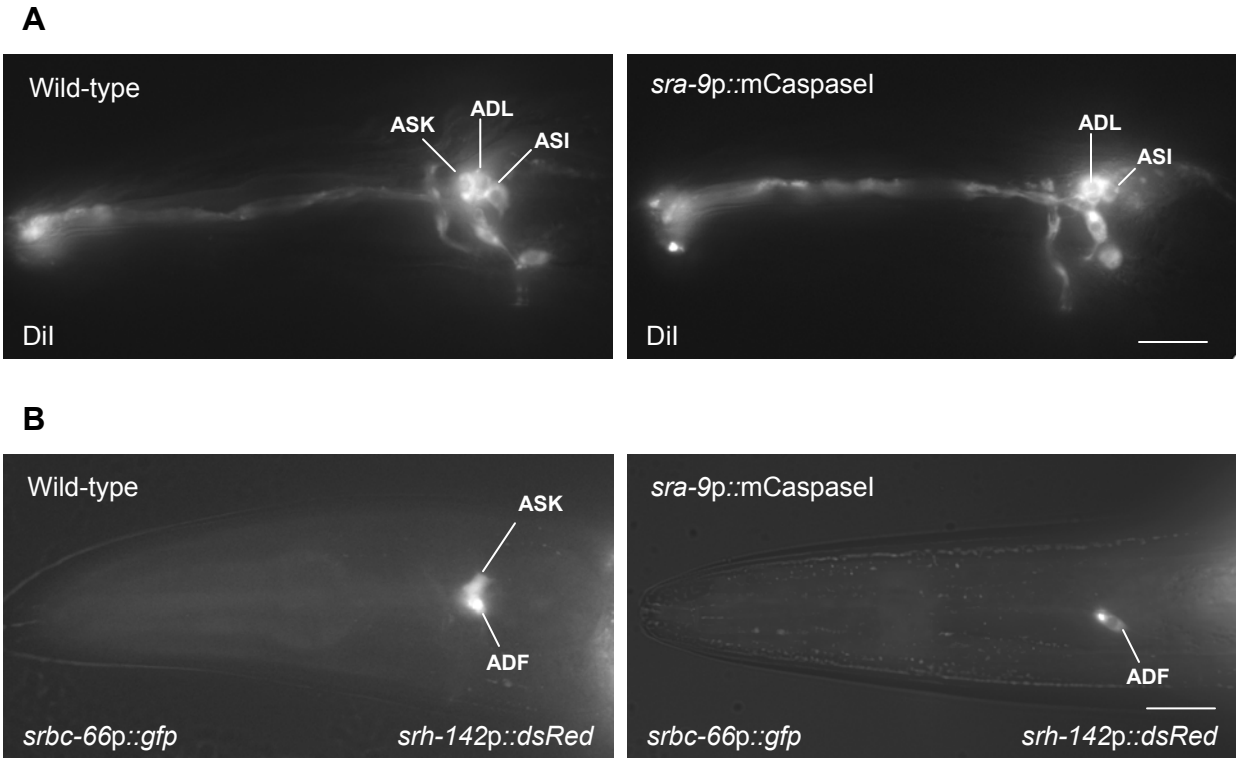


**Fig. s7.** The ASK neurons are absent in strains expressing mCaspase1 under an ASK-specific promoter. **(A)** The ASK neurons are one of six neuron types that take up lipophilic dyes (*1, 2*). No dye-filled ASK neurons are observed in animals expressing mCaspase1 under the ASK-specific *sra-9* promoter (*11*). **(B)** The *srbc-66*p*::gfp* and *srh-142*p*::dsRed* fusion genes are expressed in the ASK and ADF neurons, respectively (*12*). Expression in the ASK neurons is abolished in animals expressing *sra-9*p::mCaspase1. Lateral view; scale – 10 μm.

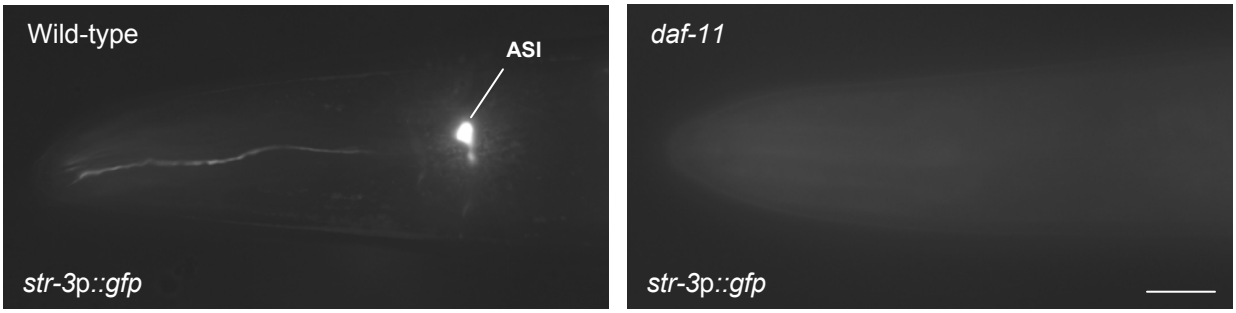


**Fig. s8.** Mutations in the *daf-11* receptor guanylyl cyclase gene downregulate *str-3*p*::gfp* expression. The *daf-11(sa195)* allele was used. Lateral view; scale – 10 μm.

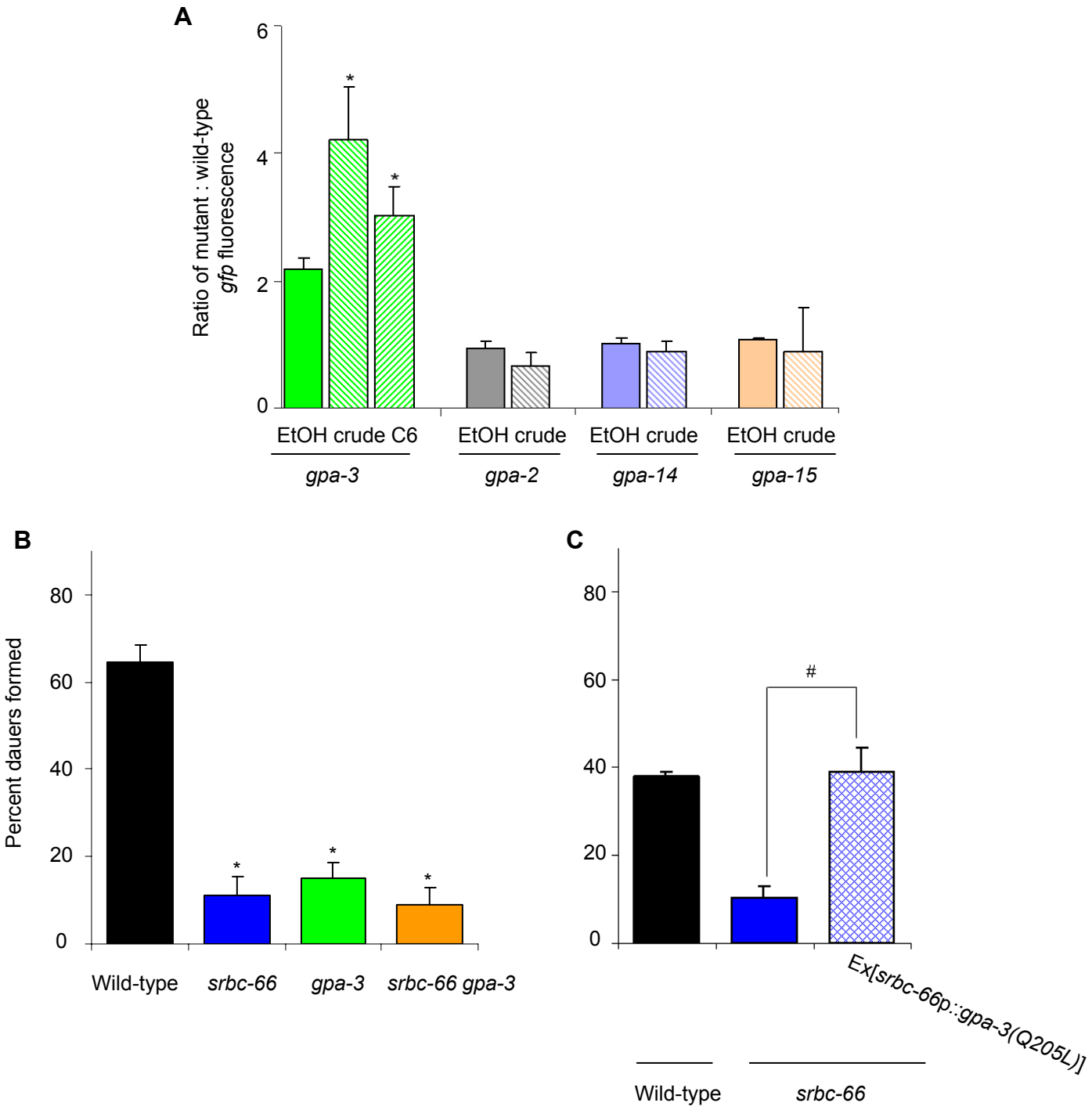


**Fig. s9.** The GPA-3 Gα subunit mediates pheromone signaling in the ASK neurons. **(A)** Mutations in *gpa-3*, but not other ASK-expressed Gα subunit genes (*13*) affect pheromone-mediated downregulation of *str-3*p*::gfp* expression. n > 50; 2 independent experiments each. **(B-C)** Percent dauers formed when animals of the indicated genotypes were grown in the presence of 600 nM **(B)** and 60 nM C6 **(C)**, respectively. Shown are the averages from at least 4 independent experiments with 50-100 animals each. # indicates values that are different at $P < 0.01$ between the values indicated by brackets. * indicates values that are different at $P < 0.01$ from corresponding values of wild-type animals.

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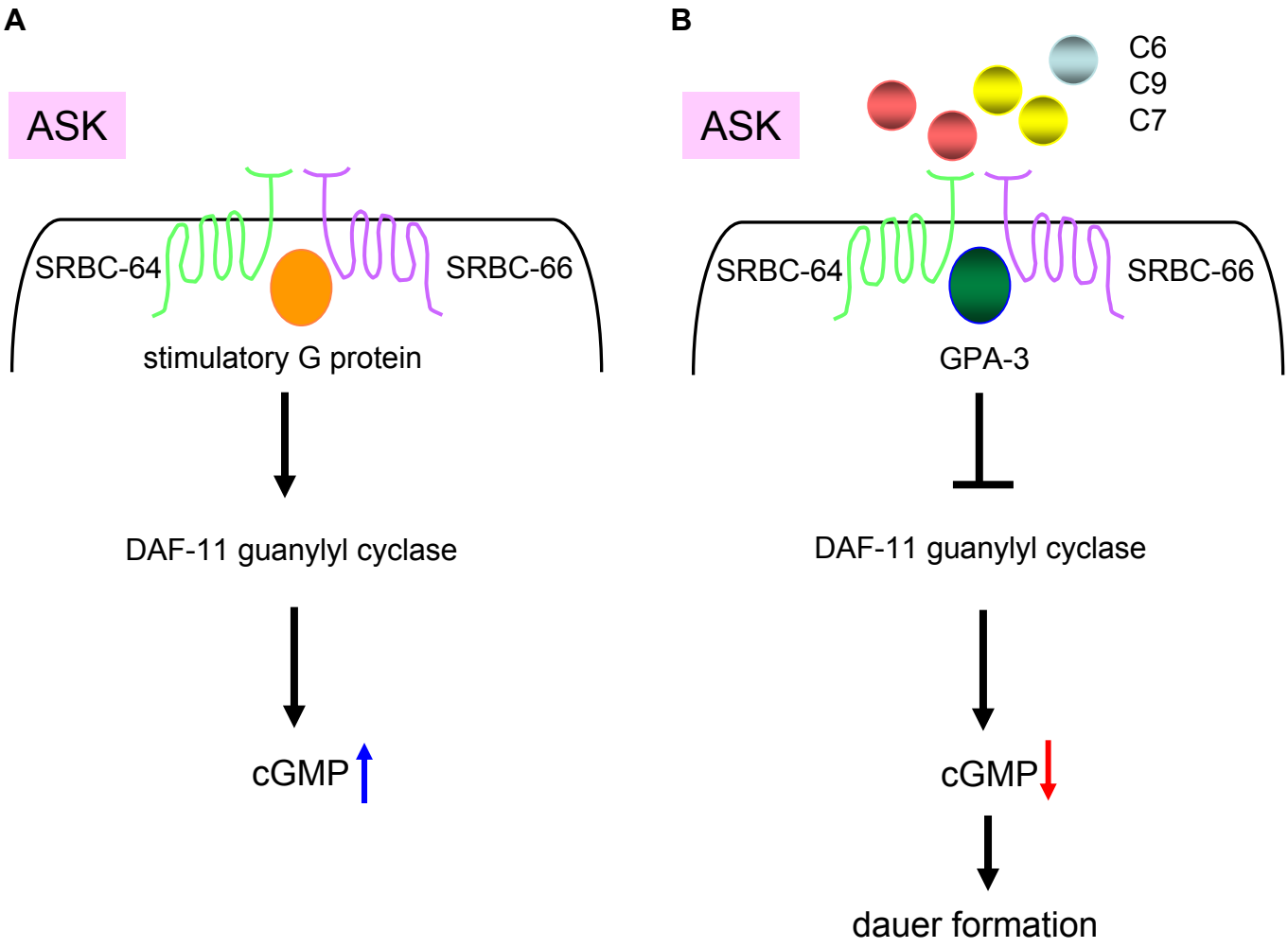


**Fig. s10.** Working model of pheromone-mediated signal transduction in the ASK neurons. **(A)** In the absence of pheromone, SRBC-64 and SRBC-66 may couple to a stimulatory Gα protein to increase intracellular cGMP levels via activation of the DAF-11 receptor guanylyl cyclase. **(B)** Upon pheromone addition, the receptors may now couple with the GPA-3 Gαi/o-like protein to inhibit DAF-11 activity and decrease intracellular cGMP levels. Decreased intracellular cGMP levels may be necessary for the ASK neurons to send a dauer-promoting signal to downstream cells including the ASI neurons. GPCRs such as the β-adrenergic receptor have previously been shown to couple to either Gαs or Gαi, based on phosphorylation state and intracellular cAMP levels (*14, 15*). Other models are also possible.

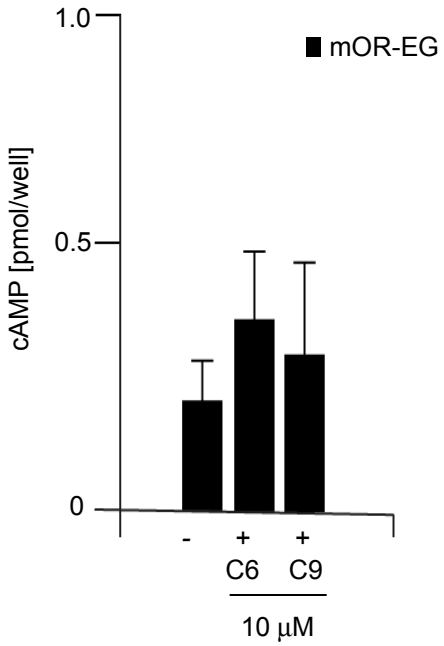


**Fig. s11.** C6 and C9 do not affect eugenol-mediated cAMP increases via the mouse eugenol OR in heterologous cells. HEK293 cells expressing the mouse eugenol olfactory receptor (mOR-EG) were stimulated with 1 mM eugenol in the absence or presence of 10 μM C6 and C9. n = 4.

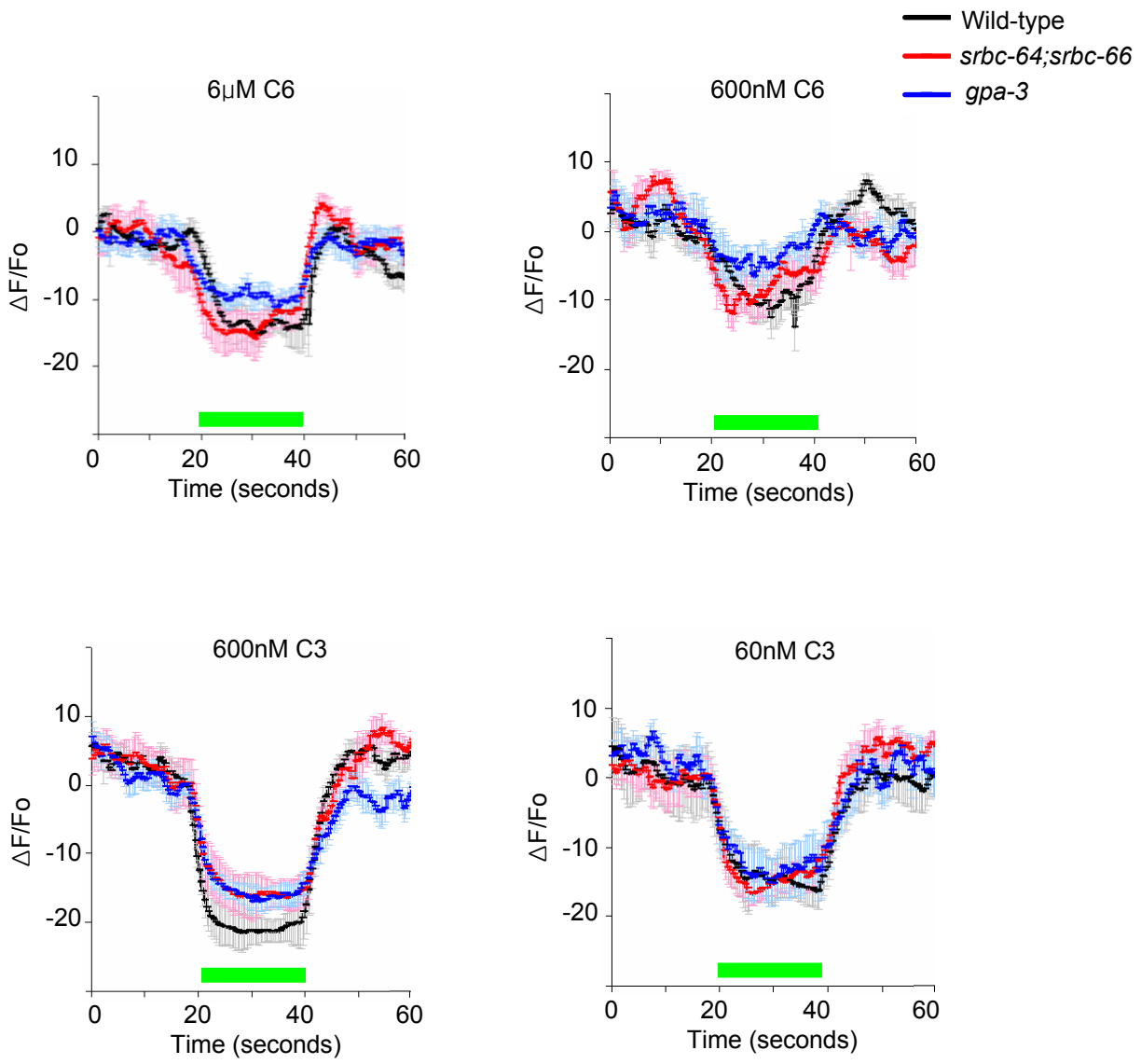


**Fig. s12.** C6 and C3 do not affect pheromone-induced G-CaMP signals in the ASK neurons of adult hermaphrodites. Average G-CaMP signals in the ASK neurons of adult animals of the indicated genotypes in response to addition of C6 or C3 (green bars). Shown are the average responses of at least 10 animals each.

In addition to downregulating *daf-7* TGF-β signaling, pheromone also downregulates insulin signaling via the *daf-2* insulin receptor (*16*). Thus, both *daf-7* and *daf-2* mutants constitutively enter into the dauer stage. *srbc-66; daf-7* and *srbc-66; daf-2* double mutants exhibited dauer formation phenotypes similar to those of *daf-7* or *daf-2* single mutants alone (table s1), consistent with the notion that SRBC-66 acts upstream of TGF-β and insulin signaling to regulate dauer formation.

**Table s1.** SRBC-66 acts upstream of TGF-β and insulin signaling to regulate dauer formation.

| Strain | Percent dauers formed (n) |
|---|---|
| Wild-type | 0 (100) |
| *srbc-66(tm2943)* | 0 (142) |
| *daf-7(e1372)* | 100 (106) |
| *daf-7(e1372); srbc-66(tm2943)* | 100 (155) |
| *daf-2(e1370)* | 100 (103) |
| *daf-2(e1370); srbc-66(tm2943)* | 100 (122) |

## REFERENCES


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