The Importance of cGMP Signaling in Sensory Cilia for Body Size Regulation in *Caenorhabditis elegans*

Manabi Fujiwara,*[,1] Takahiro Hino,* Ryuta Miyamoto,* Hitoshi Inada,[†] Ikue Mori,[‡] Makoto Koga,* Koji Miyahara,[§] Yasumi Ohshima,*[,2] and Takeshi Ishihara*

*Department of Biology, Graduate School of Science, Kyushu University, Fukuoka, 819-0395, Japan, [†]Department of Developmental Neuroscience, Tohoku University Graduate School of Medicine, Miyagi 980-8575, Japan, [‡]Department of Biological Sciences, School of Science, Nagoya University, Nagoya 464-8602, Japan, and [§]Department of Applied Life Science, Faculty of Biotechnology and Life Science, Sojo University, Kumamoto 860-0082, Japan

ABSTRACT The body size of *Caenorhabditis elegans* is thought to be controlled by sensory inputs because many mutants with sensory cilium structure defects exhibit small body size. The EGL-4 cGMP-dependent protein kinase acts in sensory neurons to reduce body size when animals fail to perceive sensory signals. In addition to body size control, EGL-4 regulates various other behavioral and developmental pathways, including those involved in the regulation of egg laying and chemotaxis behavior. Here we have identified *gcy-12*, which encodes a receptor-type guanylyl cyclase, as a gene involved in the sensory regulation of body size. Analyses with GFP fusion constructs showed that *gcy-12* is expressed in several sensory neurons and localizes to sensory cilia. Genetic analyses indicated that GCY-12 acts upstream of EGL-4 in body size control but does not affect other EGL-4 functions. Our studies indicate that the function of the GCY-12 guanylyl cyclase is to provide cGMP to the EGL-4 cGMP-dependent kinase only for limited tasks including body size regulation. We also found that the PDE-2 cyclic nucleotide phosphodiesterase negatively regulates EGL-4 in controlling body size. Thus, the cGMP level is precisely controlled by GCY-12 and PDE-2 to determine body size through EGL-4, and the defects in the sensory cilium structure may disturb the balanced control of the cGMP level. The large number of guanylyl cyclases encoded in the *C. elegans* genome suggests that EGL-4 exerts pleiotropic effects by partnering with different guanylyl cyclases for different downstream functions.

KEYWORDS body size; *Caenorhabditis elegans*; guanylyl cyclase; cGMP-dependent protein kinase; sensory cilia

THE perception of sensory cues allows an animal to respond appropriately to the environment and to adapt to changing conditions. The *Caenorhabditis elegans* hermaphrodite has 302 neurons, 60 of which are ciliated sensory neurons. Through the cilia of sensory neurons, *C. elegans* perceives taste, odor, osmotic change, and pheromones. Similar to the cilia of sensory neurons in other organisms, the sensory cilia in *C. elegans* are specialized structures where various signaling components, including sensory receptors and G proteins, are localized (Silverman and Leroux 2009).

The importance of cilia-based sensory perception in *C. elegans* development and behavior has been demonstrated by analyses of a class of mutants that lack a normal cilium structure (Perkins *et al.* 1986). These mutants, such as *che-2*, show reduced responses to taste, odor, osmotic change, and pheromones (Lewis and Hodgkin 1977; Bargmann *et al.* 1993; Fujiwara *et al.* 1999). Further analyses of these mutants also indicated that normal sensory cilia are required for the regulation of various important aspects of *C. elegans* development and physiology, including body size, locomotory pattern, life span, and fat storage (Apfeld and Kenyon 1999; Fujiwara *et al.* 2002; Alcedo and Kenyon 2004; Mak *et al.* 2006). Interestingly, sensory cues were also shown to regulate life span in *Drosophila* (Libert *et al.* 2007), indicating evolutionarily conserved roles of sensory perception through cilia.

Adult body size of *che-2* and other cilium defective mutants showed a 20–30% decrease compared with that of the wild-type animal (Fujiwara *et al.* 2002; Fujiwara *et al.* 2010). Several other mutants with defects in sensory perception

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[1]Corresponding author: Department of Biology, Graduate School of Science, Kyushu University, 744, Motooka, Nishi-ku Fukuoka, 819-0395. E-mail: fujiwara.manabi.734@m.kyushu-u.ac.jp
[2]Present address: Kyushu University, 1-17-16 Midorigaoka, Higashiku, Fukuoka 813-0021.

were also reported to exhibit small body size (Kuhara *et al.* 2002; Lanjuin and Sengupta 2002). A screen for suppressor mutants of small body size due to a *che-2* mutation revealed an important role of a cGMP-dependent protein kinase (PKG), EGL-4, in body size regulation (Fujiwara *et al.* 2002). Mutations in *egl-4* result in a large-body phenotype and completely suppress the small-body phenotype of the cilium structure mutant (Fujiwara *et al.* 2002; Hirose *et al.* 2003). This suggests that EGL-4 acts to reduce body size, and that cilia-dependent signal(s) keep body size normal by inhibiting EGL-4 activity. *egl-4* is expressed in several neurons and other tissues. Although the *egl-4* expression in muscle, hypodermis, and intestine has a certain rescuing effect, the expression in sensory neurons is sufficient for normal body size regulation (Fujiwara *et al.* 2002; Nakano *et al.* 2004). The mechanism by which EGL-4 PKG activity in sensory neurons affects body size is unknown. Genetic analysis suggests that several components in TGF-β signaling pathways, including the DBL-1/TGF-β ligand and DAF-3/Smad transcription factor, are involved in body-size regulation downstream of EGL-4 (Daniels *et al.* 2000; Fujiwara *et al.* 2002; Nakano *et al.* 2004). EGL-4 also regulates gene expression by antagonizing a histone deacetylase (van der Linden *et al.* 2008). Another study has shown that EGL-4 affects ploidy in hypodermal cells (Tain *et al.* 2008). While the precise mechanism is unknown, this accumulating evidence suggests that under the control of sensory inputs, EGL-4 regulates body size by affecting humoral factors through transcriptional regulation and alteration of ploidy in somatic tissues.

The mechanism by which EGL-4 kinase activity is controlled by sensory inputs is also unclear. Since EGL-4 kinase activity is dependent on cGMP (Stansberry *et al.* 2001; Hirose *et al.* 2003), it is likely provided by one or more of the guanylyl cyclases encoded in the *C. elegans* genome. A total of 34 guanylyl cyclases (GC) have been identified in the *C. elegans* genome, of which 7 are soluble cyclases and 27 are receptor-type cyclases (Ortiz *et al.* 2006). Compared with other species, the number of receptor-type GCs encoded in the *C. elegans* genome is unusually large. For instance, the *Drosophila* genome contains 6 and mammalian genomes contain 7 (Morton 2004). The GCs in *C. elegans* are expressed in different subsets of neurons and/or tissues such as muscle and intestine (Ortiz *et al.* 2006), suggesting a distinct function for each GC. For example, ODR-1 and DAF-11 receptor-type GCs are expressed in many chemosensory neurons and control opening of the TAX-4 cyclic-nucleotide-gated channel, thereby playing indispensable roles in chemosensation (Birnby *et al.* 2000; L'Etoile and Bargmann 2000). However, the GCs involved in the EGL-4 activation upstream of body size regulation have not been identified.

EGL-4 seems to be a multifunctional regulator with apparently unrelated functions, because *egl-4* mutations cause not only the body size phenotype but also a wide variety of phenotypes including defects in the control of egg laying, dauer formation, fat storage, chemosensory behavior, locomotory state, life span, sleep-like state, and even satiety signaling (Trent *et al.* 1983; Daniels *et al.* 2000; L'Etoile *et al.* 2002; Hirose *et al.* 2003; Raizen *et al.* 2006; Raizen *et al.* 2008; You *et al.* 2008; Hao *et al.* 2011; O'halloran *et al.* 2012). Therefore, EGL-4 activity upstream of each of these functions is likely to be controlled separately in *C. elegans*.

In this article, we report a new suppressor mutation of the small-body phenotype of the *che-2* mutant, whose responsible gene encodes a guanylyl cyclase, GCY-12. Analyses of *gcy-12* mutants revealed that only some EGL-4 functions require GCY-12, while other functions may require different guanylyl cyclases. We also found that a cyclic nucleotide phosphodiesterase, PDE-2, acts as another EGL-4 regulator of body size control. Our findings point to the importance of cGMP signaling in sensory body size regulation and suggest a complex mechanism underlying the control of cGMP-dependent kinase activity.

Materials and Methods

Strains and genetics

Wild-type animals were the *C. elegans* strain N2. Worms were grown at 20° with food using standard methods (Brenner 1974).

Strains used in this work included *gcy-12(ks99)*, *gcy-12(ks100)*, *gcy-12(nj10)*, *che-2(e1033)*, *egl-4(ky185)*, *egl-4(n478)*, *daf-11(m47)*, *odr-1(n1936)*, and *pde-2(qj6)*. Double mutants were generated using standard methods and were confirmed by complementation tests or by sequencing. *che-2;Ex[che-2/H20::gfp]* was provided by Dr I. Katsura, Nationai Institue of Genetics, Japan (Fujiwara *et al.* 1999).

The *pde-2(qj6)* mutant was generated by Tc1 transposon insertion and subsequent imprecise excision, which caused deletion of a 1129-bp coding region containing exons 3-5 of *pde-2* and the insertion of an extra 132 bp of sequence originating from the Tc1 transposon. Because of a stop codon in the inserted Tc1 sequence, the *pde-2(qj6)* locus is expected to produce a truncated protein lacking both the GAF domain and the catalytic domain of PDE. A 9-kb genomic DNA fragment containing the *pde-2* gene and its 3.0-kb-upstream and 0.5-kb-downstream regions was amplified by PCR and used for a rescue experiment.

Body size measurement

L4 crescent stage animals were transferred to 6-cm seeded NGM plates and maintained in a well-fed condition until body size measurement. For body volume measurement, animals were anesthetized with NaN_3 48 hr after the L4 stage and measured using 'Senchu-gazou-kaiseki-souchi SVK-3A' (Showa Denki Co., Fukuoka, Japan) as described by Hirose *et al.* (2003). Each data point represents the average volume of >20 animals. All strains compared in each graph were cultured and measured at the same time/condition.

Screen for che-2 small-body-size suppressors (Chb), mapping, and cloning of gcy-12

che-2(e1033) animals were mutagenized with 50 mM ethyl methanesulfonate (EMS) using the standard protocol (Brenner 1974) and 40,000 F2 progenies (from ~3000 F1 progenies)

were screened for animals that exhibited increased body size but that still retained a ciliary defect. *ks100* was mapped using the single nucleotide polymorphism (SNP) method (Wicks *et al.* 2001), based on a suppressor phenotype of the reduced body size and confined tracking pattern of *che-2*. The locus was mapped to a ~120-kb region (between snip markers on the cosmids C54A12 and EEED8) around the center of LGII. Among the cosmid clones covering this area, ZK622 and F08B1 showed rescuing activities; *che-2(e1033);ks100* double mutant animals exhibited a decrease in body size when either of the cosmids was introduced. ZK622 and F08B1 are adjoining cosmids and only one gene, *gcy-12*, exists in the overlapping region. A genomic fragment containing the whole coding region and 3 kb upstream of the predicted start site of the *gcy-12* gene also rescued the body size phenotype of *ks100*.

Dye-filling assay and other behavioral assays

Dye-filling assay: assays were performed as described by Starich *et al.* (1995) using DiI (10 μg/ml). More than 30 animals per strain were observed.

Chemotaxis: assays were performed as described by Bargmann *et al.* (1993). Each data point represents the average chemotaxis index of 9–14 chemotaxis plates.

Adaptation to benzaldehyde: assays were performed as described by L'Etoile *et al.* (2002). To adapt and test animals, benzaldehyde at 1/50000 and 1/300 dilution were used, respectively. Each data point represents the average chemotaxis index of 8–11 chemotaxis plates.

Counting eggs in the uterus: single young adult animal (27 hr after the L4 stage) was bleached and the eggs left behind were counted. Twenty-six to 36 animals per strain were counted.

Dauer formation: eggs were gathered by bleaching well-fed adults and placed on seeded plates (200–300 eggs/plate). After 50 hr at 27°, the numbers of dauers and non-dauers were counted. The criteria for dauer formation were larvae with a long body shape, dark intestine, nonpumping pharynxes, and lethargic movements. Each data point represents the average dauer rate of 11–14 culture plates.

Plasmid construction

genomic gcy-12::gfp: A genomic *gcy-12*::*gfp* construct was made by inserting an 8.1-kb genomic fragment containing a 3-kb promoter region and the entire coding region of *gcy-12* into pPD95.77 (GFP expression vector; a gift from Dr A. Fire, Stanford University) in frame using *Xba*I and *Msc*I sites. The stop codon was substituted to a *Bal*I site by PCR so that GFP was fused to the C terminus of the GCY-12 protein.

gcy-12 expression construct for rescue and overexpression: A *gcy-12* expression construct was made by inserting an 8.1-kb genomic fragment containing a 3-kb promoter region and the entire coding region of *gcy-12* into pPD49.26 (expression vector; a gift from Dr A. Fire) using *Xba*I and *Kpn*I sites.

odr-1p::gcy-12::gfp, srb-6p::gcy-12::gfp, tax-4p::gcy-12::gfp, ceh-36p::gcy-12::gfp, gpa-9p::gcy-12::gfp, dat-1p::gfp constructs: First, the GCY-12::GFP expression vector was made by inserting a *gcy-12* cDNA (obtained from cDNA clone, yk316d1, provided by Dr Y. Kohara, National Institute of genetics, Japan; the 5′ part was supplemented with a product of 5′ RACE) into pPD95.77 in frame so that GFP was fused to the C terminus of the GCY-12 protein. Then, to generate different promoter-*gcy-12* constructs, a 2.4-kb *odr-1* promoter region, a 3.3-kb *srb-6* promoter region, a 3-kb *tax-4* promoter region, a 3.1-kb *ceh-36* promoter region, a 2.8-kb *gpa-9* promoter region, or a 1.4-kb *dat-1* promoter region were inserted into the GCY-12::GFP expression vector.

gcy-12p::gcy-12::gfp and gcy-12p::gcy-12 (ΔECD)::gfp: *gcy-12p*::*gcy-12*::*gfp* was made by inserting a 3-kb *gcy-12* promoter region into the GCY-12::GFP expression vector. For *gcy-12p*::*gcy-12* (ΔECD)::*gfp*, the coding region for the 442-amino acid (aa) extracellular domain of *gcy-12* cDNA was deleted.

Generation of transgenic worms

Transgenic strains were generated by standard micro-injection methods (Mello *et al.* 1991). Unless otherwise noted, test DNA was injected at 100 ng/μl with *myo-3*::*gfp* DNA at 33 ng/μl (a GFP construct expressed in body wall muscle) or *lin-44*::*gfp* at 33 ng/μl (a GFP construct expressed in hypodermal cells at the tip of the tail) or *ttx-3*::mRFP at 33 ng/μl (an mRFP construct expressed in AIY neurons) as a co-injection marker. Generally we isolated multiple (two to five) independent transgenic lines from each injection and confirmed that there were no major differences among them. For *gcy-12* overexpression analysis, the plasmid was injected into N2 animals at 150 ng/μl with the co-injection marker, *sra-6*::*gfp* (33 ng/μl). To compare the effect of *gcy-12* overexpression in different genetic backgrounds, the same extrachromosomal arrays were transferred by mating from N2 to *egl-4(ky185)* and to *egl-4(n478)*.

Data availability

All strains and plasmids described here and in the Supporting Information are available upon request.

Results

Identification of new Chb mutations, ks99 and ks100

To identify new genes involved in the sensory body size regulation, we performed a Chb (**ch**e-2 small-**b**ody-size suppressors) screen because a previous screen was apparently not saturated (Fujiwara *et al.* 2002). We mutagenized *che-2(e1033)* animals by EMS treatment and looked for animals with an increased body size. In the 6000 haploid-genome scale screening, we isolated six candidate lines (*X;che-2*) (Figure 1). All six of these lines still demonstrated the dye-filling defect, indicating that they still retained the *che-2* mutation.

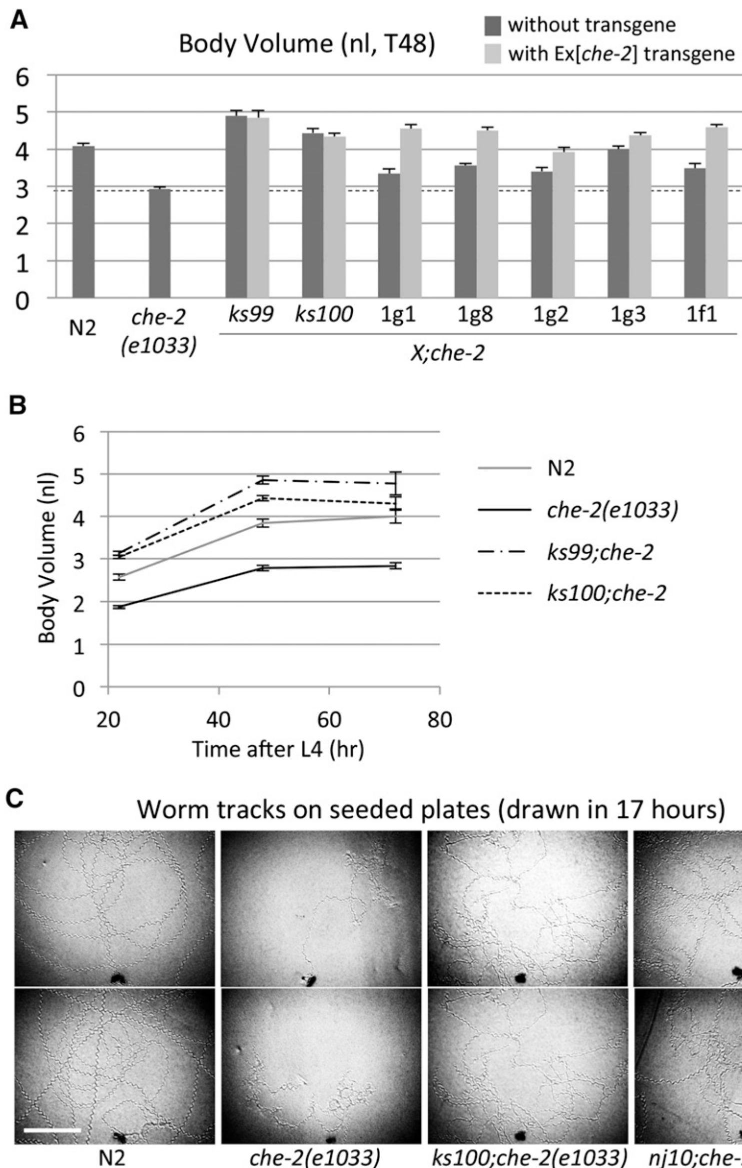


Figure 1 The mutants identified in a Chb (*che-2* small-body suppressor) screen. (A) The adult stage (48 hr after the L4 stage) body volumes of the *che-2(e1033)* mutant and of seven suppressor mutants, x;*che-2 (e1033)*. The *che-2* gene (Ex[*che-2*]) was introduced into each suppressor mutant to test whether it increased body size further (class II suppressors) or whether body size remained unchanged (class I suppressors). *ks99* and *ks100* were classified as class I and the other mutants were classified as class II. (B) Time-course analyses of *ks99* and *ks100* body size. Body volumes were measured at the indicated time points following the L4 stage. (C) Worm tracking patterns on an *E. coli* lawn. A single worm was left on a seeded plate for 17 hr and allowed to move freely. Two examples of each strain are shown. Scale bar, 5 mm. Error bars indicate SEMs.

Next, we introduced a *che-2* transgene (*Ex[che-2]*) into each candidate line (*X;che-2*) to rescue the *che-2* locus. If the animals with the transgene (*X;che-2;Ex[che-2]*) exhibited the same body size as those without the transgene (*X;che-2*), we classified the line into class I. Class I suppressor genes presumably act downstream of *che-2* dependent sensory inputs for body size regulation and hence rescue of the *che-2* mutation is expected to have no effect on the body size of class I suppressor mutants. In contrast, if the animals with the transgene exhibit an increase in body size relative to control animals without the transgene, we classified the line into class II. Since the effects of *che-2* and the suppressor mutation are additive, class II suppressor genes are likely to act in an independent pathway parallel to the *che-2* pathway. Out of the six mutations, two (*ks99* and *ks100*) were classified as class I suppressors, while the other four were classified as class II (Figure 1).

Interestingly, we found that *ks99* and *ks100*, but none of the class II mutations, suppressed the confined tracking pattern of *che-2* animals (Figure 1 for *ks100*, data not shown for *ks99*, see below for *nj10*). This is a common characteristic of class I mutations, including *egl-4* (Fujiwara *et al.* 2002), indicating that the body size and the locomotory pattern are controlled by the same molecular mechanism.

Both ks99 and ks100 are mutations in gcy-12

ks99 and *ks100* mutations were linked to linkage group II (LGII); therefore, we performed a complementation test and found that *ks99* and *ks100* could not complement each other's Chb phenotype (data not shown), suggesting that they are mutations in the same gene. Subsequently, *ks100* was used for further mapping to identify the responsible gene. By SNP mapping, we have narrowed the mutation site to a ~120-kb region (between snip markers on cosmids

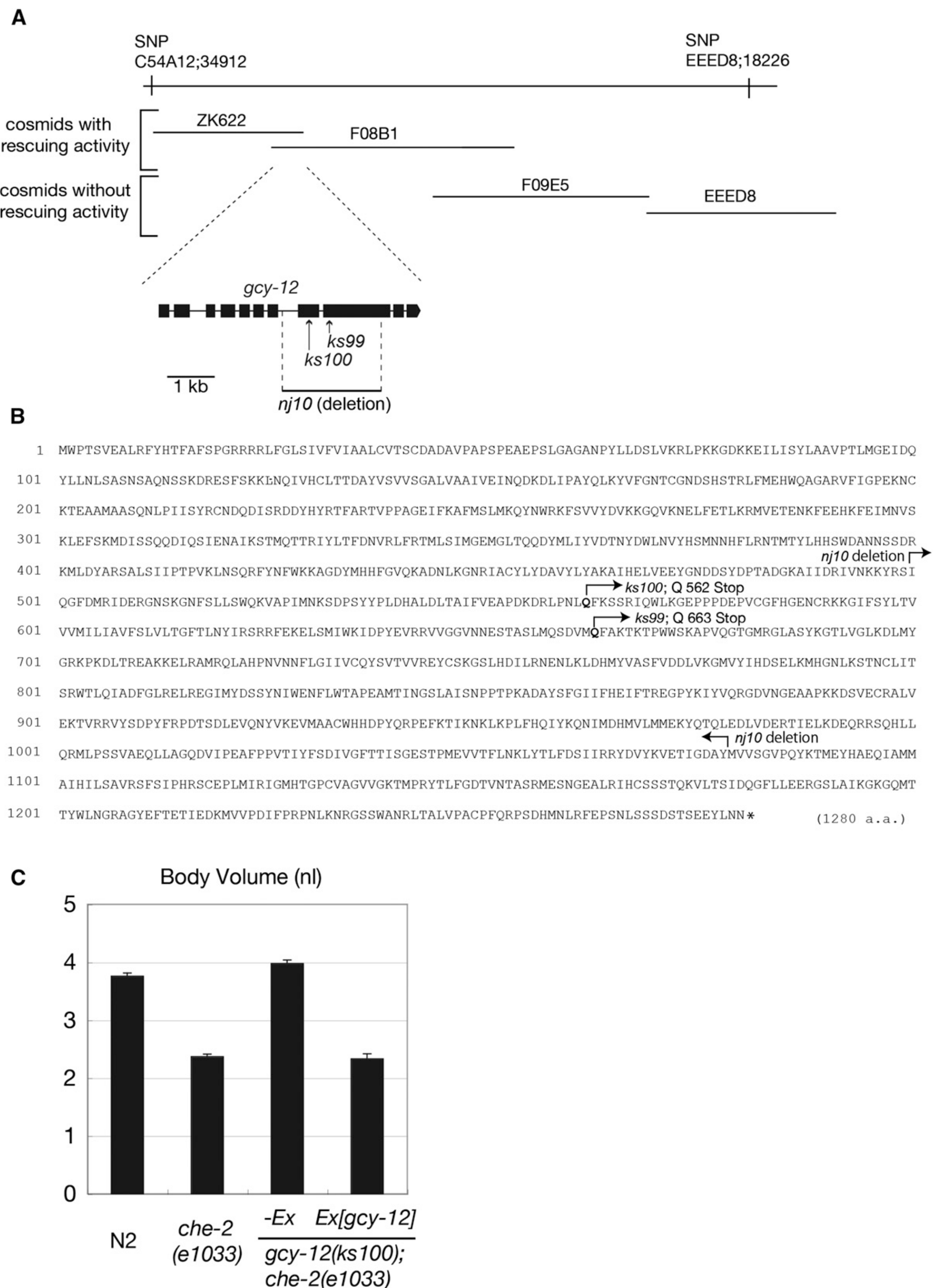


Figure 2 *gcy-12* is a new Chb mutant. (A) Genetic and physical maps of *ks100*. SNP mapping placed *ks100* in a ~120-kb region around the center of LGII. Two cosmids, ZK622 and F08B1, rescued *ks100*. Only one gene, *gcy-12* was encoded in the overlap region of the two cosmids. (B) *gcy-12* encodes a 1280-aa receptor-type guanylyl cyclase. The amino acid sequence was predicted from a cDNA obtained by RT–PCR and an EST clone, yk316d1. Mutation sites in *ks99* and *ks100* and the amino acid sequence deleted in *nj10* are shown. (C) The transgene containing the entire *gcy-12* coding region (Ex[*gcy-12*]) reduced the body size of the *gcy-12(ks100);che-2(e1033)* mutant to that of the *che-2(e1033)* mutant. Body volumes at the adult stage (48 hr after the L4 stage) are shown. Error bars indicate SEMs.

C54A12 and EEED8) that is near the center of LGII. We introduced several cosmids covering the area into *che-2(e1033);ks100* double mutant animals and examined the rescuing activity of each cosmid, the rescue phenotype being a decrease in body size. Two adjoining cosmids, ZK622 and F08B1, showed a rescue, and only one gene, *gcy-12*, exists in the overlapping region. Because general toxicity of introduced DNA can reduce body size, we first sequenced the *gcy-12* locus of *ks100* and *ks99* animals for mutations. As expected, both *ks100* and *ks99* had mutations in the coding region of *gcy-12*, which are predicted to result in early termination of translation (Figure 2, A and B). The genomic fragment containing the whole coding region and 3 kb upstream of the predicted start site showed rescuing activity of the Chb phenotype (Figure 2C).

gcy-12 encodes a receptor-type guanylyl cyclase. It consists of an extracellular ligand-binding domain, a transmembrane domain, a kinase-like domain, and a guanylyl cyclase catalytic domain. The nonsense mutations in *ks99* and *ks100* are predicted to result in GCY-12 proteins that lack all intracellular domains, including the catalytic domain, indicating that both are likely to be null alleles. To further characterize *gcy-12* mutants, we also analyzed a deletion allele, *nj10*, obtained by the TMP/UV method (Gengyo-Ando and Mitani 2000). *nj10* has a 2197-bp deletion removing exon 8 and part of exon 9 (Figure 2, A and B). The deleted region corresponds to the transmembrane domain, the kinase-like domain, and about half of the guanylyl cyclase domain; therefore, the *nj10* allele represents a null allele. *gcy-12(nj10)* suppressed the small body size of the *che-2* mutant in the same manner as *ks100* and *ks99* (Supporting Information, Figure S1).

Genetic interaction between gcy-12 and egl-4

A key regulator of the sensory body-size regulation is the cGMP-dependent protein kinase EGL-4. Mutations in *egl-4* result in the class I Chb phenotype and the expression of *egl-4* cDNA in several sensory neurons can rescue this phenotype (Fujiwara *et al.* 2002). The EGL-4 kinase is activated by cGMP (Stansberry *et al.* 2001; Hirose *et al.* 2003); therefore, it is conceivable that cGMP produced by the GCY-12 guanylyl cyclase activates EGL-4 *in vivo*. To examine this possibility by genetic analyses, we first made a *gcy-12;egl-4* double mutant. For the *egl-4* mutant, we mainly used *egl-4(ky185)* because it has a 777-bp deletion that removes the coding sequence corresponding to a major part of the kinase domain and is therefore likely a null allele (Fujiwara *et al.* 2002). The *gcy-12*, as well as the *egl-4* mutant, shows an increased body size compared with wild-type animals (N2) (Figure 3 and Figure S2). However, the *gcy-12;egl-4* double mutant did not become larger than the *egl-4* single mutant, indicating that *gcy-12* and *egl-4* act in the same genetic pathway. However, we noted that the body size of the *egl-4* mutant is larger than that of the *gcy-12* mutant, suggesting that GCY-12 is not the only guanylyl cyclase involved in EGL-4 activation for body-size regulation.

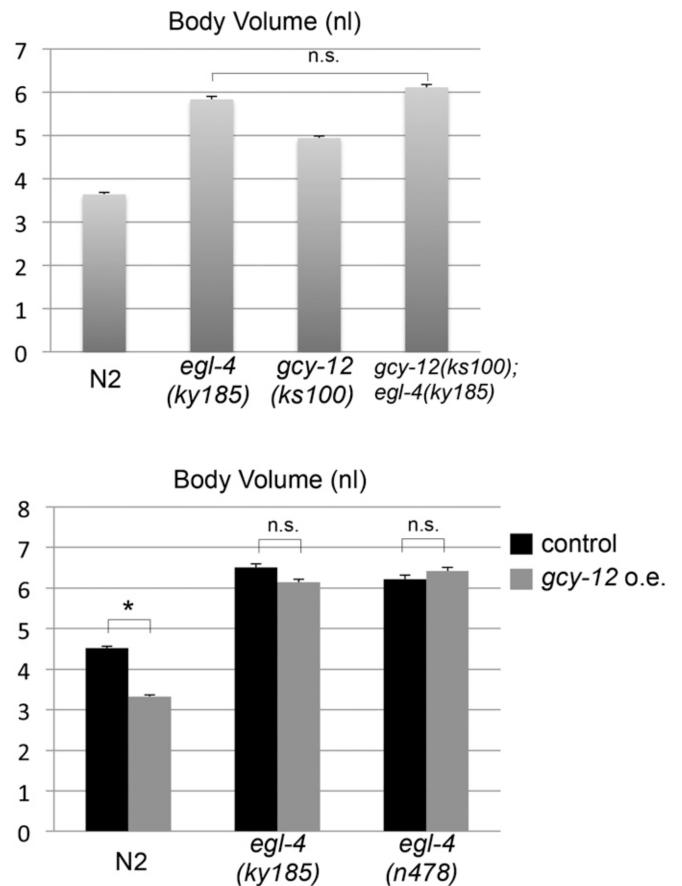


Figure 3 GCY-12 is likely to act through the EGL-4 kinase for body-size regulation. The body volumes at the adult stage (48 hr after the L4 stage) are shown for the indicated strains. Top: *gcy-12(ks100);egl-4(ky185)* double mutant was not bigger than the *egl-4(ky185)* single mutant. Bottom: *gcy-12* overexpression (o.e.) reduced the size of the wild-type animal, but did not affect the body size of the *egl-4(ky185)* or *egl-4(n478)* mutants. Error bars indicate SEMs (n.s., not significant; *, $P < 0.001$; *t*-test).

To confirm that GCY-12 acts through EGL-4 activation, the effect of GCY-12 overexpression was examined. We introduced a genomic fragment containing the *gcy-12* gene and its promoter region into wild-type and *egl-4* animals. The overexpression of GCY-12 in wild-type animals led to a small body size (Figure 3). This appears to result from inadequate activation of the EGL-4 kinase in wild-type animals, because the GCY-12 overexpression did not cause body-size reduction in the *egl-4(ky185)* mutant (Figure 3). We confirmed this result with another *egl-4* mutant, *n478* (Figure 3). Together, these results suggest that GCY-12 acts upstream of EGL-4, possibly by providing cGMP to the kinase.

Characterization of a gcy-12 mutant

EGL-4 cGMP-dependent kinase is involved in the regulation of several aspects of behavior and development, as well as the regulation of body size and locomotory pattern. As shown above, *gcy-12* mutants and *egl-4* mutants share the suppressor phenotype of the small body and confined tracking pattern of the *che-2* mutant, and GCY-12 seems to act upstream

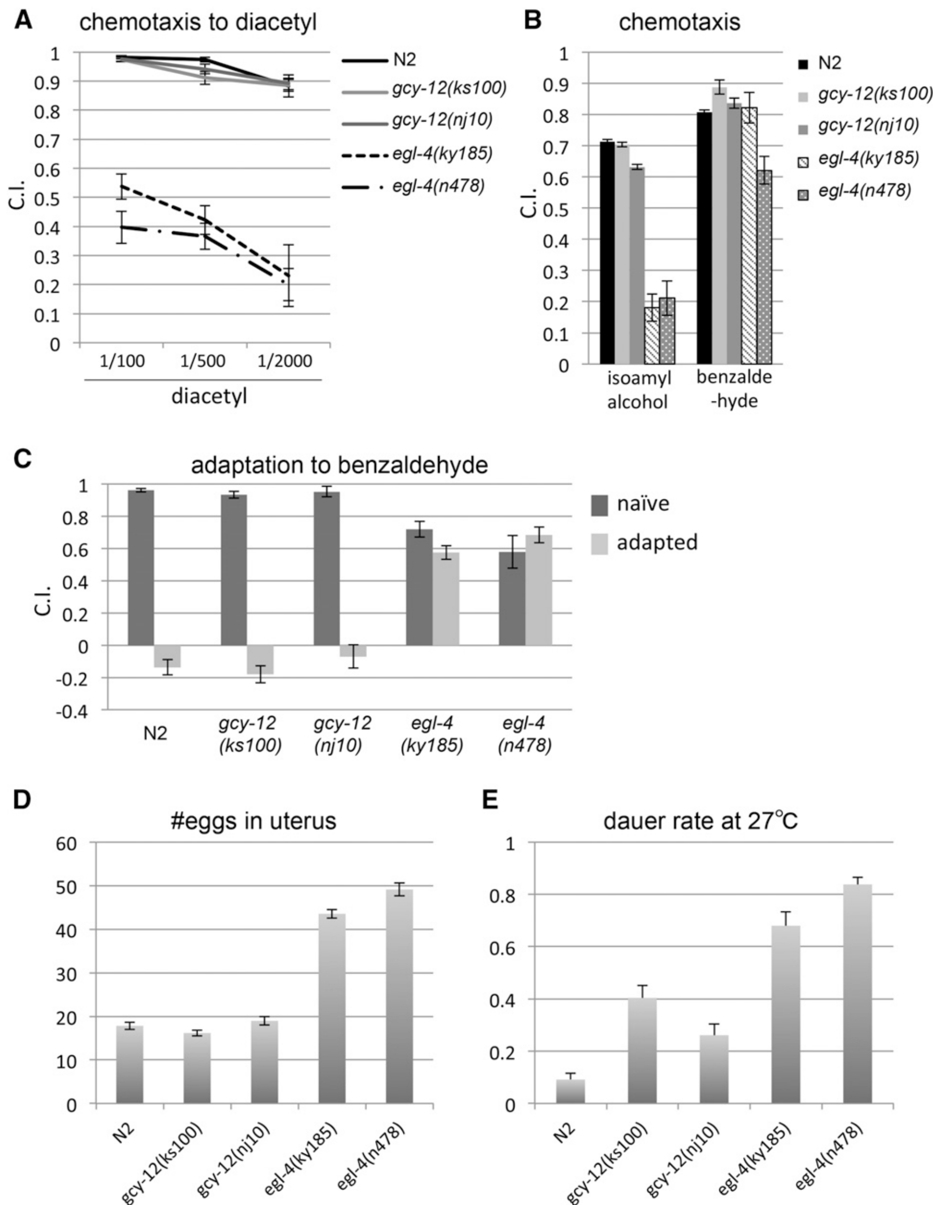


Figure 4 Mutant phenotypes of *gcy-12*. The *gcy-12* mutants do not share all phenotypes exhibited by the *egl-4* mutants. (A and B) Chemotaxis indexes to diacetyl (1/100, 1/500, 1/2000 dilutions), isoamyl alcohol (1/1000 dilution), and benzaldehyde (1/500 dilution) are shown. (C) Chemotaxis indexes to benzaldehyde (1/300 dilution) prior to and after conditioning (exposure to 1/50000 diluted benzaldehyde for 90 min) are shown. (D) The average number of eggs in an adult (27 hr after the L4 stage). (E) Dauer formation rates in culture conditions at 27°. Error bars indicate SEMs.

of EGL-4, at least for body-size regulation. Therefore, we next examined if *gcy-12* mutants share the other phenotypes of *egl-4* mutants, including egg-laying, chemotaxis, and odorant adaptation defects and inadequate dauer formation (Daniels *et al.* 2000). *egl-4* mutants showed chemotaxis defects in responding to the volatile attractants, diacetyl and isoamylalcohol, but retained relatively high responsiveness to another volatile attractant, benzaldehyde (Figure 4, A and B), as previously reported (Daniels *et al.* 2000). On the other hand, *gcy-12(ks100)* and *gcy-12(nj10)* mutants showed almost normal responses to these three odorants, exhibiting a different chemotaxis trait from *egl-4* mutants (Figure 4, A and B). *egl-4* mutants also showed an adaptation defect to benzaldehyde (L'Etoile *et al.* 2002). *gcy-12(ks100)* and *gcy-12(nj10)* mutants showed normal adaptation, while *egl-4* mutants retained high responsiveness to benzaldehyde after preexposure (Figure 4C). Next, we examined the egg-laying phenotype. While *egl-4* mutants accumulated 40–50 eggs in the uterus, the egg retention of *gcy-12(ks100)* and of *gcy-12(nj10)* was comparable to wild-type animals (∼20) (Figure 4D). Another phenotype of *egl-4* is the formation of dauer larvae, an alternative developmental stage of diapause, under noninducing conditions (Daniels *et al.* 2000). *gcy-12(ks100)* and *gcy-12(nj10)* mutants showed higher dauer rates than N2 under those conditions (Figure 4D). *egl-4* mutants, however, showed much higher dauer rates than N2 and *gcy-12* mutants (Figure 4D). These results indicated that not all phenotypes of *egl-4* mutants are shared by *gcy-12* mutants. Therefore, guanylyl cyclases other than GCY-12 may activate EGL-4, at least for the regulation of chemotaxis, odorant adaptation, and egg laying. In terms of the control of dauer formation, GCY-12 might have a role in EGL-4 activation.

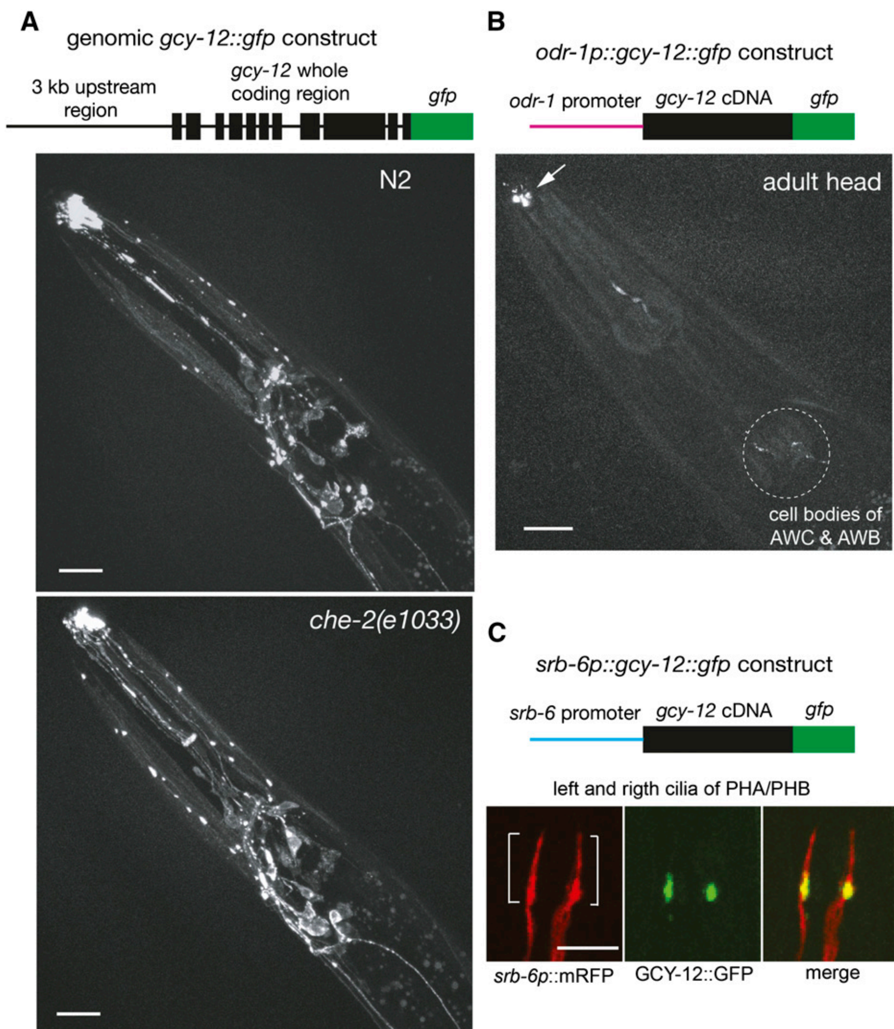
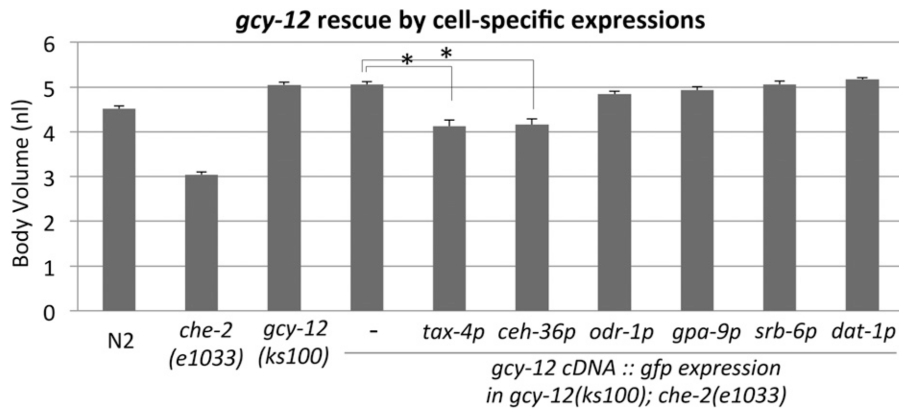


Figure 5 Expression analyses of *gcy-12*. Schematic of *gfp* expression constructs and the projections of confocal images are shown. An LSM510 confocal microscope (Carl Zeiss) was used to scan GFP and mRFP fluorescence. The nose tip is to the left (A and B). (A) The expression patterns of *genomic gcy-12*::*gfp* in the head regions of wild-type and *che-2(e1033)* animals at the L4 stage. Scale bar, 20 μm. (B) The expression patterns of *odr-1p*::*gcy-12*::*gfp* in the head region of a wild-type animal at the adult stage. The arrow indicates the dendrite tip and the dashed circle indicates cell bodies of AWC and AWB neurons. Scale bar, 20 μm. (C) The localization patterns of GCY-12::GFP in the sensory cilia of PHA/PHB neurons in the tail. The cilia (brackets) were visualized with *srb-6p*::*mRFP*. Scale bar, 5 μm.

In addition to the phenotypes shown by *egl-4* mutants, we examined the *gcy-12* mutant for other phenotypes. Based on the visualization of several sensory neurons by fluorescent proteins, *gcy-12(nj10)* did not exhibit any apparent defects in neuronal morphology, including sensory cilia (Figure S3). Differentiation markers of sensory neurons, including *sra-6p*::*gfp*, *srb-6p*::mRFP, *odr-1p*::*gfp*, *gcy-5p*::*gfp*, *gcy-7p*::*gfp*, and *str-2p*::*gfp*, were expressed correctly in *gcy-12(nj10)*, indicating no apparent defect in the differentiation of the following neurons: ASH, ASI, PHA, PHB, AWB, AWC, ASE, and AWC^{on} (Troemel *et al.* 1995; Troemel *et al.* 1999) (data not shown).

gcy-12 is expressed in sensory neurons

The GCY-12 expression pattern was determined by fusing GFP to the C terminus of GCY-12 in a genomic fusion (genomic *gcy-12*::*gfp*). This construct was made by inserting *gfp* into a genomic construct that included the whole coding region of *gcy-12* and a 3-kb upstream region. We confirmed that the genomic *gcy-12*::*gfp* construct retained rescue activity (Figure S4). This clone directs GFP expression in chemosensory neurons (AWC, ASE, ASJ, AUA, PHA, PHB), an interneuron (PVQ), several other unidentified head neurons, an excretory gland cell, and adult head muscles (Figure 5A). In the head muscles, GFP shows a punctate pattern. The strongest GFP expression was observed at the tip of the nose, which appeared to be sensory cilia. To observe clear subcellular localization in sensory neurons, we drove the expression of GFP-tagged GCY-12 only in subsets of chemosensory neurons. *gfp* was ligated to the 3′ terminus of a *gcy-12* cDNA and expressed under the control of an *odr-1* promoter (for expression in AWB and AWC chemosensory neurons) or an *srb-6* promoter (for expression in PHA and PHB chemosensory neurons) (Troemel *et al.* 1995; L'Etoile and Bargmann 2000) (Figure 5, B and C). With *odr-1p*::*gcy-12*::*gfp*, we observed a strong localization of GFP around the distal dendrite tip of AWC and AWB neurons. We also observed weak GFP expression in the cell bodies of these neurons as small dots around the nuclei, apparently reflecting transport through the Golgi apparatus (Figure 5B). With *srb-6p*::*gcy-12*::*gfp*, the relatively simple morphology of the cilia of PHA and PHB neurons allowed us to observe GCY-12 enriched mostly at the proximal segment of cilia or at the distal dendrite tip that is just close to cilia (Figure 5C).



promoter	expression cells
tax-4p	AWB, **AWC**, AFD, **ASE**, ASG, ASI, **ASJ**, ASK, URX, BAG
ceh-36	**AWC, ASE**
odr-1p	AWB, **AWC**
gpa-9p	**ASJ**, **PVQ**, **PHB**
srb-6p	ADL, ADF, ASH, **PHA**, **PHB**
dat-1p	ADE, PDE, CEP

(bold: cells in which the expression of genomic *gcy-12::gfp* was observed)

Figure 6 GCY-12 acts in a set of sensory neurons. *gcy-12 cDNA::gfp* was expressed under the control of various sensory promoters in *gcy-12(ks100);che-2(e1033)* animals, and the body volumes were measured at the adult stage (48 hr after the L4 stage). Error bars indicate SEMs. (*, $P < 0.01$; *t*-test with Bonferroni correction). The table shows the cellular expression patterns induced by each promoter.

We observed GFP expression in several different tissues; therefore, we next sought to determine the identity of the cells in which GCY-12 acts to control body size. We hypothesized that expression in sensory neurons is important, because it was shown that the Chb phenotype of *egl-4* animals can be rescued by sensory expression of *egl-4* under a *tax-4* promoter (Fujiwara *et al.* 2002). Therefore, we examined if the expression of *gcy-12* under a *tax-4* promoter can rescue the Chb phenotype of *gcy-12* animals. As shown in Figure 6, we observed that the expression of *gcy-12* cDNA, which was fused to *gfp*, under the *tax-4* promoter can reduce the body size of the *gcy-12;che-2* double mutant (Figure 6). This finding suggests that GCY-12 and EGL-4 act in the same sensory neurons to control body size and is consistent with the predicted role of GCY-12 in the activation of EGL-4. Furthermore, we tried further rescue experiments using an additional five promoters that promote expression in reduced subsets of the sensory neurons. Among them, we found that *gcy-12* expression in AWC and ASE neurons under a *ceh-36* promoter reduced the body size to a similar level as that produced by expression under the *tax-4* promoter (Figure 6). Because *gcy-12* expression in AWC (and AWB) under an *odr-1* promoter did not have a significant effect, the expression of *gcy-12* in ASE or in both neurons, ASE and AWC, seems to be important for the body size regulation. The expression of *gcy-12* in other sensory neurons under *gpa-9*, *srb-6*, and *dat-1* promoters did not show any significant effects on body size.

Genetic analyses of the relationship between GCY-12 and other GCs

In *C. elegans*, 27 receptor-type guanylyl cyclase genes have been identified in the complete genome sequence (Ortiz *et al.* 2006). Some are known to function in sensory neurons by activating cyclic nucleotide-gated channels encoded by *tax-2* and *tax-4* (Coburn and Bargmann 1996; Komatsu *et al.* 1996; Inada *et al.* 2006; Ortiz *et al.* 2009; Hallem *et al.* 2011; Murayama *et al.* 2013). For example, two guanylyl cyclase genes, *daf-11* and *odr-1*, play a key role in regulating chemotaxis in AWC and ASE chemosensory neurons (Birnby *et al.* 2000; L'etoile and Bargmann 2000). Our analyses suggest that GCY-12 acts in *tax-4* expressing neurons, particularly in ASE and possibly in AWC, to activate EGL-4 and regulate body size. Therefore, we asked if the other GCs in *tax-4*-expressing neurons, such as DAF-11 and ODR-1, are also involved in sensory-dependent body size regulation. First, we examined if a *daf-11* or *odr-1* mutation exhibits the Chb phenotype in a *che-2(e1033)* background. *daf-11(m47)* caused a significant but small increase in the body size of *che-2(e1033)* animals, while *odr-1(n1936)* did not (Figure 7). Next, we examined if loss of DAF-11 or ODR-1 enhances the *gcy-12* body size phenotype. While a *gcy-12(ks100)* mutant exhibits a large body size, *daf-11(m47)* and *odr-1(n1936)* mutants exhibit a normal body size (Figure 7). We predicted that double mutants would be larger than the *gcy-12* single mutant, if ODR-1 and DAF-11 can complement the supply of cGMP in the absence of GCY-12. We found that *gcy-12(ks100);daf-11(m47)* becomes even smaller than each single mutant (Figure 7). This might reflect detrimental effects due to the specific combination of these two mutations. On the other hand, *gcy-12(ks100);odr-1(n1936)* was larger than each single mutant (Figure 7). Taken together, ODR-1 may partially complement GCY-12 for body-size regulation, possibly through supplying cGMP to the EGL-4 kinase in the absence of GCY-12, while DAF-11 seems to have little effect on the regulation of body size.

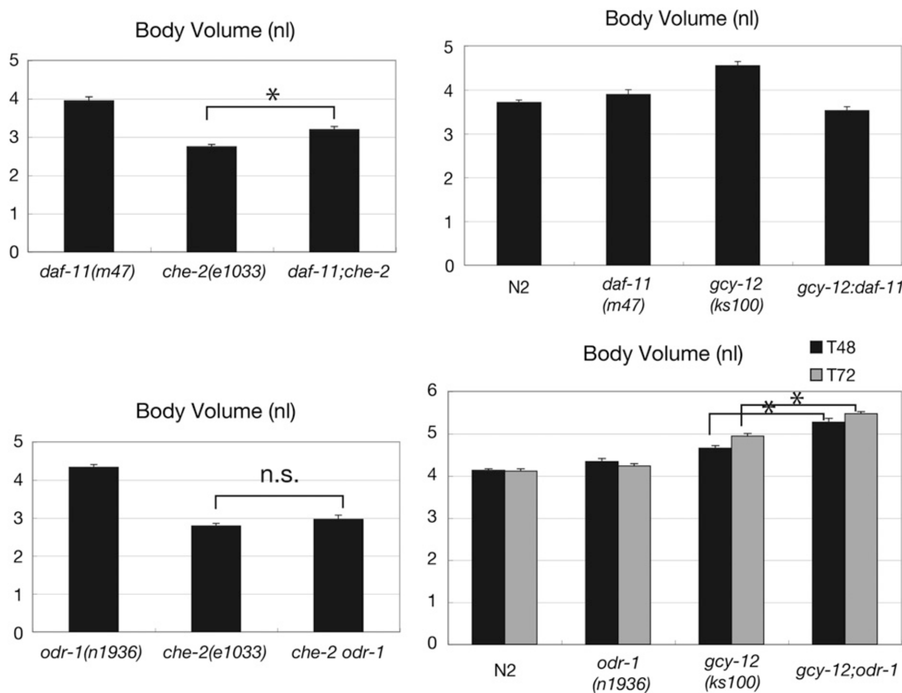


Figure 7 Genetic interaction analyses of *daf-11(m47)* and *odr-1(n1936)* with the body-size mutants, *che-2(e1033)* and *gcy-12(ks100)*. *daf-11(m47)* and the compared strains were cultured at 15° to prevent dauer entry and the body volumes were measured at the adult stage (99 hr from the L4 stage). *odr-1(n1936)* and the compared strains were cultured at 20° as usual and the body volumes were measured at the adult stage (48 and 72 hr after the L4 stage). Error bars indicate SEMs (n.s., not significant; *, $P < 0.001$; *t*-test).

Role of the extracellular domain of GCY-12

GCY-12 has an extracellular domain that is conserved from bacteria to mammals (Ortiz *et al.* 2006). The domain is termed RFLBR (receptor family ligand-binding region) and is speculated to be required for ligand binding. We next sought to determine if the RFLBR is required for the control of GCY-12 activity. A large part of the GCY-12 extracellular domain (442 aa) was deleted and this truncated construct was introduced into the *gcy-12(ks100)* mutant. As shown in Figure 8, the increased body size of *gcy-12* animals was rescued by the truncated form of GCY-12. The effect is comparable to that of full-length GCY-12. This result indicates that the extracellular domain is not required for the activation of GCY-12.

Possible role of PDE-2 for the regulation of EGL-4 in controlling body size

The intracellular cGMP level is known to be tightly controlled both by its rate of synthesis by GCs and by its rate of hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). By RNAi and/or mutant analyses of the six PDE homologs in *C. elegans* (Charlie *et al.* 2006), we found that the *pde-2* gene is required for normal body size regulation (Figure 9 and data not shown). *pde-2* encodes the closest *C. elegans* homolog of human cGMP-dependent 3′,5′-cyclic phosphodiesterase 2A (PDE2A). Human PDE2A is stimulated by cGMP binding to its GAF domain and hydrolyzes both cGMP and cAMP (Omori and Kotera 2007). *C. elegans* PDE-2 retains the conserved phosphodiesterase catalytic domain and the GAF domain. The *pde-2(qj6)* mutation, a deletion allele that is expected to produce a truncated protein lacking the whole catalytic domain (see *Materials and Methods*), caused a small-body-size phenotype (Figure 9), and the phenotype was rescued by introduction of the wild-type *pde-2* gene (Figure S5). Importantly, the *pde-2(qj6)* mutation did not affect the body size of the *egl-4(ky185)* mutant, indicating PDE-2 action upstream of EGL-4 (Figure 9). The *gcy-12(ks100);pde-2(qj6)* double mutant exhibited an intermediate body size relative to each single mutant (Figure 9). These results indicate that PDE-2 antagonizes GCY-12 and negatively regulates the EGL-4 kinase in controlling body size.

The *pde-2(qj6)* animals retain fewer eggs in the uterus than wild-type animals, and the phenotype can be rescued by introduction of the wild-type *pde-2* gene (Figure 9). This phenotype, termed Egl-c (egg-laying constitutive), is opposite to the egg-accumulation phenotype (Egl-d; egg-laying defective) exhibited by the *egl-4(ky185)* loss-of-function mutant. Since an *egl-4 (ad450)* gain-of-function mutant also shows the Egl-c phenotype (Raizen *et al.* 2006), PDE-2 is likely to negatively regulate the EGL-4 kinase in egg-laying control. The *pde-2(qj6)* mutation did not decrease the number of eggs retained in the uterus in the *egl-4(ky185)* background (*pde-2;egl-4* in Figure 9), supporting the role of PDE-2 upstream of EGL-4. In the regulation of chemotaxis behavior, PDE-2 appears to play a dispensable role; the *pde-2(qj6)* mutant showed normal responsiveness to all the attractive odorants examined, and the *pde-2(qj6);egl-4 (ky185)* double mutant showed the same chemotactic defects as were observed for the *egl-4(ky185)* single mutant (Figure 9).

Discussion

To investigate the mechanism of sensory-dependent regulation of body size in *C. elegans*, we performed a mutant screen for suppressors of *che-2* small body size and identified the

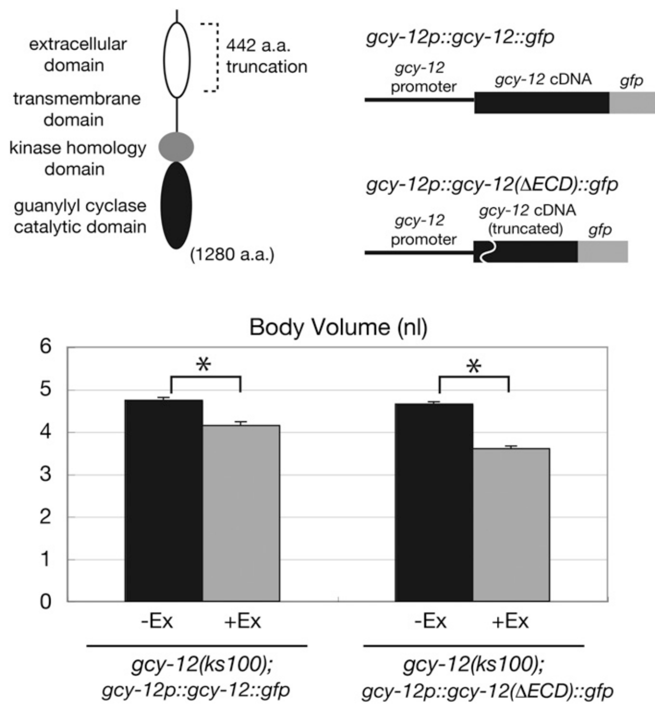


Figure 8 Extracellular domain of GCY-12 is dispensable for body size regulation. Top left: domain structure of the GCY-12 receptor-type guanylyl cyclase. Top right: schematic images of the control and deletion constructs. Each construct was introduced into *gcy-12(ks100)* animals and the body volumes of animal without (−Ex) or with (+Ex) the transgene were measured at the adult stage (48 hr after the L4 stage). Error bars indicate SEMs (*, $P < 0.001$; *t*-test).

gene, *gcy-12*, which encodes a receptor-type guanylyl cyclase. The *gcy-12* gene was previously predicted from the *C. elegans* genome sequence database (Yu *et al.* 1997; Ortiz *et al.* 2006), and biochemical analysis of *gcy-12* by expression in COS-M6 cells also demonstrated its guanylyl cyclase activity (Yu *et al.* 1997). In the current study, we report extensive analyses of the function of this gene *in vivo*.

GCY-12 is the guanylyl cyclase that controls EGL-4 kinase activity in body size regulation

C. elegans body size is controlled by the EGL-4 cGMP-dependent protein kinase, whose activity is negatively regulated by sensory inputs (Fujiwara *et al.* 2002). Here, we have identified *gcy-12*, as another gene involved in sensory-dependent regulation of body size. Similar to *egl-4* mutations, mutations in *gcy-12* increased body size and suppressed the small-body phenotype of the *che-2* cilium-defective mutant. The *gcy-12* mutations, as well as the *egl-4* mutations, were classified as class I suppressors, indicating that the *che-2* mutation causes the small-body phenotype through altered GCY-12 function. Biochemical analyses showed that EGL-4 indeed requires cGMP for its kinase activity (Stansberry *et al.* 2001; Hirose *et al.* 2003); therefore, the simplest interpretation is that GCY-12 is the guanylyl cyclase that provides cGMP to EGL-4 in the pathway regulating body size. The studies of this article provide several lines of evidence to support this hypothesis. First, the double-mutant analysis suggested that *gcy-12* and *egl-4* act in the same pathway. Second, the overexpression analysis of *gcy-12* indicated that GCY-12 affects body size through EGL-4. Finally, rescue of the *che-2* phenotype by *gcy-12* expression under the control of the *tax-4* promoter suggested that GCY-12 and EGL-4 act in the same set of sensory neurons.

Control of GCY-12 activity in cilia

GCY-12, together with most of the other receptor-type guanylyl cyclases in *C. elegans*, contains a conserved extracellular domain, the receptor family ligand-binding region [RFLBR (PF01094)]. The presence of this conserved domain indicates that ligand binding may control GCY-12 activity and consequently regulate body size. The distinct localization of GFP-tagged GCY-12 at sensory cilia supports this idea. However, the deletion analysis of the extracellular domain (ECD) of GCY-12 indicated that the domain is dispensable for body-size regulation. It is possible that GCY-12 heterodimerizes with other guanylyl cyclases, whose extracellular domains bind to ligands. In fact, analysis of the amino acid sequence suggested that GCY-12 may form an obligate heterodimer (Morton 2004). Another possibility is that GCY-12 does not require the ECD for its activation, because GC activity is regulated independently of signals through the ECD. We prefer this possibility, because it may explain why mutants with malformed cilia, such as *che-2*, are small. Suppression of the small body size of the *che-2* mutant by the mutations in *gcy-12* and *egl-4* indicates that the GCY-12-EGL-4 signaling cascade is inadequately activated when the cilia are malformed. We observed that GCY-12::GFP is still localized to dendrite tips in the *che-2* mutant (Figure 5A). An electron microscopy analysis showed that the distal and middle segments but not the proximal segments of cilia are missing in the *che-2(e1033)* mutant (Lewis and Hodgkin 1977). Because GCY-12::GFP is mostly enriched around the base of the cilia (Figure 5C), GCY-12 in the *che-2* animals may still be capable of activating EGL-4. One possibility is that EGL-4 kinase activity in cilia may be regulated positively by GCY-12 and negatively by other signaling molecules. If the positive regulator, GCY-12, does not require extracellular signals for activation, the ciliary defect in the *che-2* mutant might inhibit only the negative regulation signaling, leading to hyperactivation of the EGL-4 kinase and consequently resulting in the small body size phenotype.

A candidate component of the negative regulation pathway is *pde-2*, mutation of which causes the small body phenotype in an EGL-4 dependent manner, as observed for the *che-2* mutation. Although a detailed expression analysis has not been performed, *pde-2* appears to be widely expressed, including in many head neurons (Hunt-Newbury *et al.* 2007; Wang *et al.* 2013). Similar to the mechanism of phototransduction signaling, sensory inputs may activate PDE-2 and negatively regulate EGL-4 in the control of body size. It would be interesting to examine cGMP levels in the cilia of *pde-2* and *gcy-12* mutants, as well as in the malformed cilia of a *che-2* mutant. Recent developments in imaging technology have made it possible to visualize cGMP levels in neurons *in vivo*,

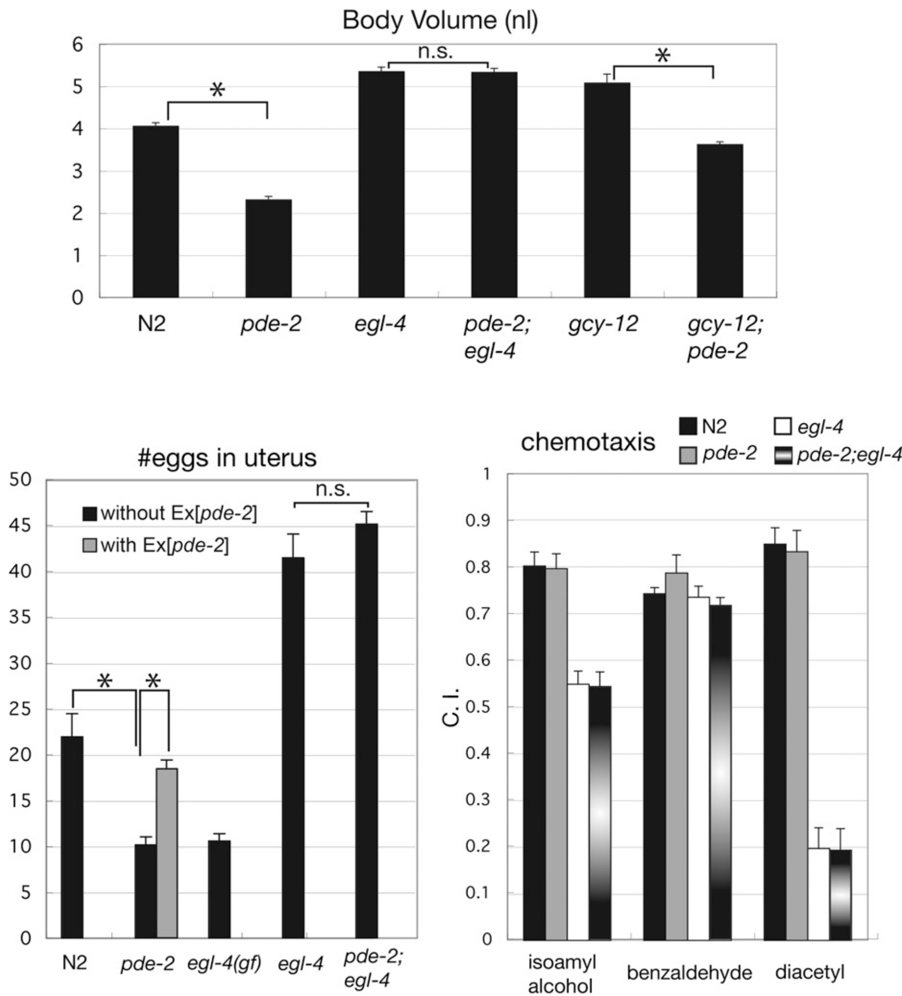


Figure 9 Phenotypes of the *pde-2(qj6)* mutant. Body volumes of wild-type, *pde-2(qj6)*, *egl-4 (ky185)*, *pde-2(qj6);egl-4(ky185)*, *gcy-12(ks100)*, and *gcy-12(ks100);pde-2(qj6)* animals were measured at the adult stage (48 hr after the L4 stage). The egg-laying phenotype and chemotaxis behavior of wild-type, *pde-2(qj6)*, *egl-4(ad450gf)*, *egl-4(ky185)*, and *pde-2(qj6);egl-4(ky185)* animals were examined. For the chemotaxis assay, isoamyl alcohol (1/100 dilution), benzaldehyde (1/500 dilution), diacetyl (1/1000 dilution) were used. The Egl-c phenotype of *pde-2(qj6)* was rescued by the transgene containing the wild-type *pde-2* gene (Ex[*pde-2*]). Error bars indicate SEMs (n.s., not significant; *, $P < 0.001$; *t*-test).

although it is still difficult to observe at subcellular levels, such as in cilia (Couto *et al.* 2013). We showed that GCY-12 expression in ASE sensory neurons is important for body-size regulation. Because ASE sensory neurons act as a main sensor for various salt ions (Bargmann and Horvitz 1991), it is possible that the salt sensation is involved in body-size regulation, for example, by affecting PDE-2 activity.

Biochemical analysis by Yu *et al.* (1997) showed that the guanylyl cyclase activity of GCY-12 is dependent on temperature, with activity higher at 25° than at 15°. Given the action of GCY-12 in body-size regulation, this suggests that animals cultivated at 25° may be smaller than those cultivated at 15°. However, we did not observe such temperature-dependent body size changes in either wild-type or *egl-4* animals (data not shown).

Function-specific regulation of EGL-4 cGMP-dependent kinase activity

EGL-4 is involved in various biological processes, including chemotaxis, odorant adaptation, dauer formation, and egg laying (Daniels *et al.* 2000). Our analyses indicated that GCY-12 is required for EGL-4 activation only in a limited number of EGL-4 functions, including body size regulation, but not in other functions including chemotaxis, odorant adaptation, and egg laying. Our analysis with the mutants of ODR-1 and DAF-11, other GCs expressed in the same neurons as GCY-12 is expressed, also suggests that these GCs do not play major roles in the body size regulation pathway. Given the existence of an unusually large number of guanylyl cyclases in *C. elegans* (~34), EGL-4 may have different GC partners for each function. This may occur through the expression of different GCs in different cells or through the localization of different GC partners in distinct functional units in the same cell. Indeed, the localization of GCY-12 to cilia is tightly controlled and its mislocalization results in the body-size defect (Fujiwara *et al.* 2010). Other GCs are also known to localize to specific subcellular compartments for compartmentalized signaling (Gross *et al.* 2014).

Using different GCs for different EGL-4 functions may enable elaborate control of the kinase. For example, in the process of odorant adaptation, activation of EGL-4 by ODR-1 is thought to induce the downregulation of ODR-1 (Juang *et al.* 2013). Without independent GC partners, such a feedback regulation would affect other processes in which EGL-4 is involved. Our study suggests that there are multiple function-specific modes of regulation of kinase activity during signal transduction for complex behavior and development.

Acknowledgments

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The Importance of cGMP Signaling in Sensory Cilia for Body Size Regulation in *Caenorhabditis elegans*

Manabi Fujiwara, Takahiro Hino, Ryuta Miyamoto, Hitoshi Inada, Ikue Mori, Makoto Koga, Koji Miyahara, Yasumi Ohshima, and Takeshi Ishihara

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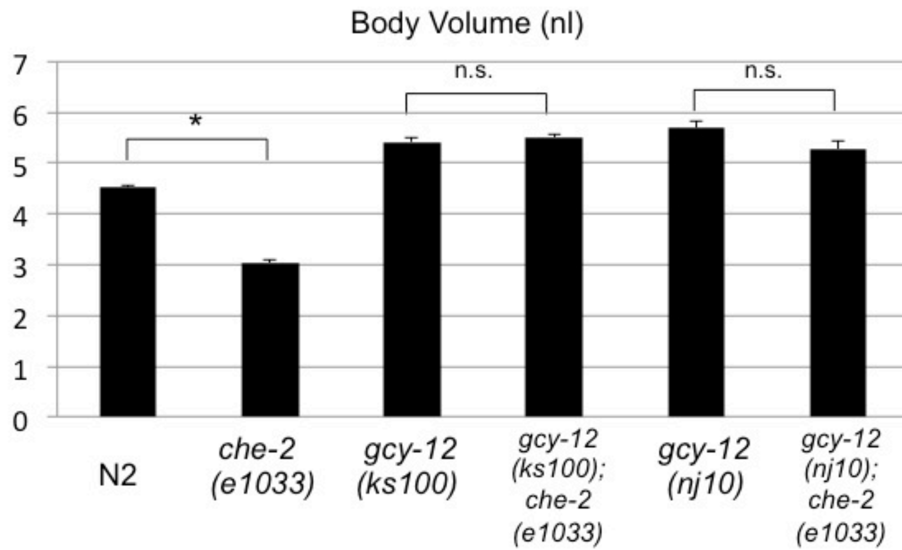

Figure S1 The body volumes at the adult stage (48 hours after the L4 stage) were shown. *gcy-12(nj10)*, as well as *gcy-12(ks100)*, suppressed the small body size phenotype induced by *che-2(e1033)* in the manner of the Class I suppressor. Error bars indicate SEMs (n.s., not significant; * $p < 0.001$; t test).

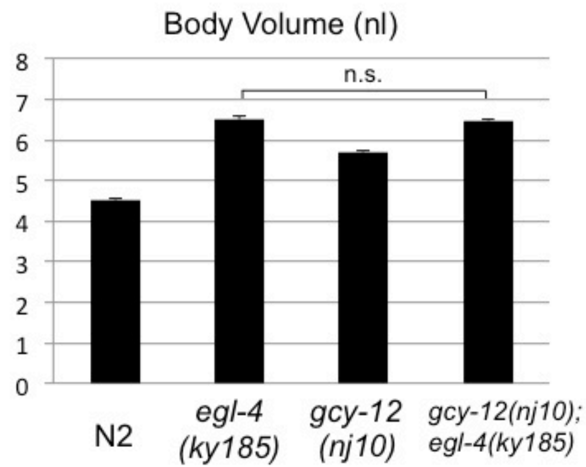

Figure S2 The body volumes at the adult stage (48 hours after the L4 stage) were shown. The *gcy-12(nj10); egl-4(ky185)* double mutant was not bigger than the *egl-4(ky185)* single mutant. Error bars indicate SEMs (n.s., not significant; t test).

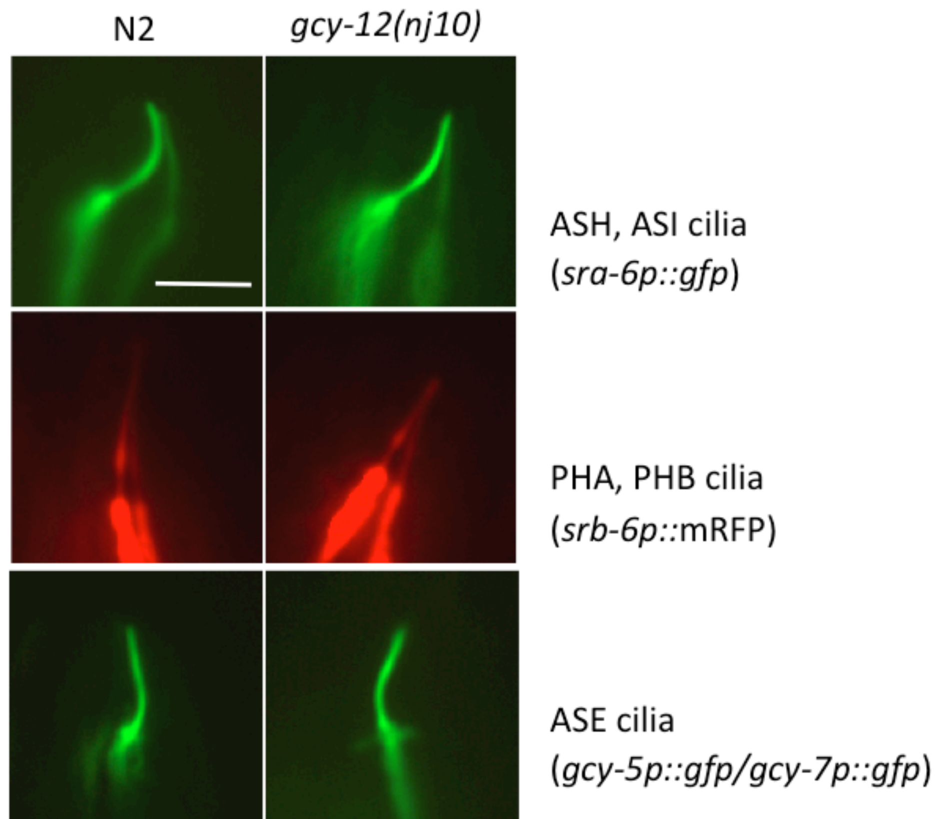

Figure S3 Cilium morphology was visualized by introducing *sra-6p::gfp* (ASI and ASH cilia) or *srb-6p::mRFP* (PHA and PHB cilia) or *gcy-5p/gcy-7p::gfp* (ASE cilia) into wild-type and *gcy-12(nj10)* animals. An Axioplan2 microscope (Zeiss) equipped with a 63x objective lens and a 3CCD camera C7780 (Hamamatsu) was used. Scale bar: 5 μm.

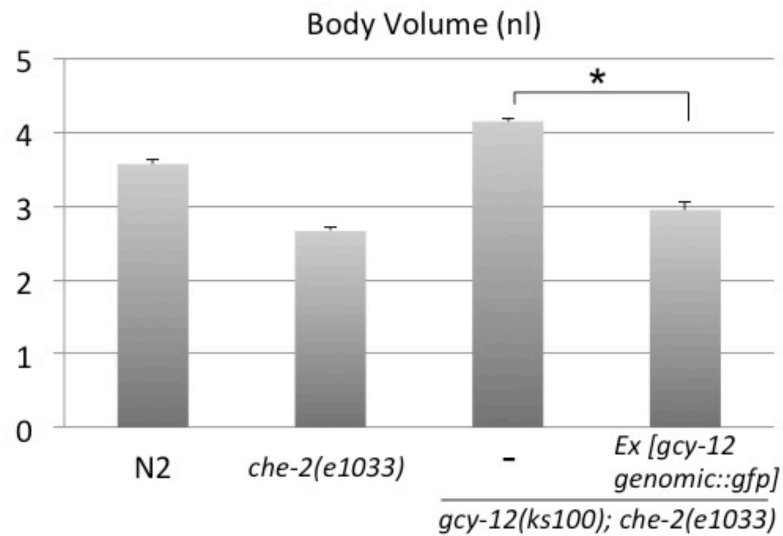

Figure S4 The genomic *gcy-12::gfp* construct had a rescuing activity. The introduction of genomic *gcy-12::gfp* into *gcy-12(ks100);che-2(e1033)* animals as an extrachromosomal array decreased the body volume. The body volumes at the adult stage (48 hours after the L4 stage) were shown. Error bars indicate SEMs (* $p < 0.001$; t test).

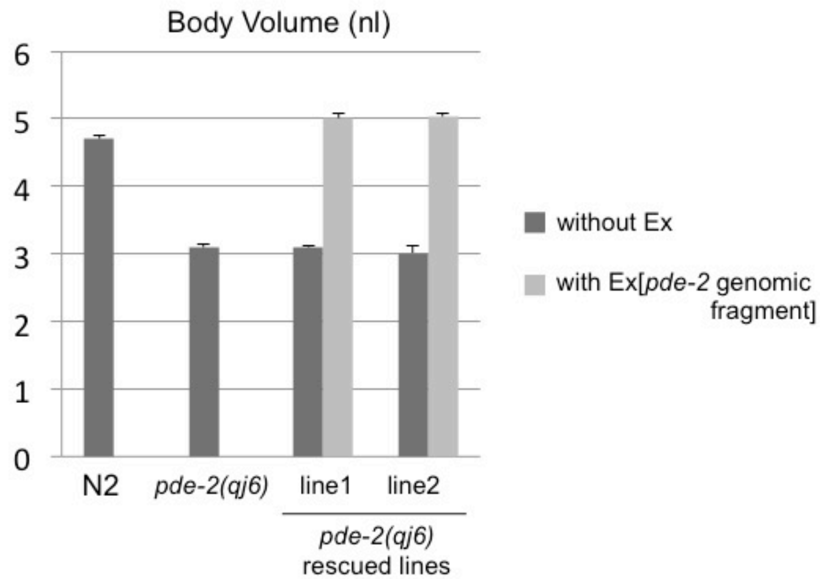

Figure S5 The small-body phenotype of *pde-2(qj6)* was rescued by introduction of the wild-type *pde-2* gene, which is obtained by PCR amplification of the coding region and its 3kb upstream and 0.5 kb downstream regions. The body volumes at the adult stage (48 hours after the L4 stage) of 2 rescued lines by the extrachromosomal arrays were shown.