EXTRAGENIC SUPPRESSORS OF HEAT SHOCK ACTIVATED GOα.

Topic I: Cyclin in Heat Shock Response

Topic II: Signaling by Go and Gq in *C. elegans*

Thesis by

Wen J. Chen

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2000

(Defended May 16th, 2000)

Truthfulness, Benevolence and Forbearance.

Acknowledgements

My advisor Paul W. Sternberg, is the one who made the whole project exist. I can never forget his continuing support throughout my time in graduate school. He showed me great motivation and encouragement, and taught me how to be a true scientist. I am also very grateful to the other advisor of mine, Melvin I. Simon, for his wonderful insight and graciousness as a scientist and a department leader. Mel allowed me to perform all the cell culture experiments in his Lab, and proved the models we proposed from *C. elegans*. There is another professor who was very important during my Ph.D. training, Henry Lester. Henry was always strict but fair with me, and I want very much to thank him, especially for making me realize the value of confidence. I also thank the other two faculties in my advisor committee, Bill Dunphy and Elliot Meyerowitz. Bill and Elliot showed great interest in my project and gave me lots of good advice every time we met.

The project is done in cooperation with Yvonne M. Hajdu-Cronin, who first taught me *C. elegans* genetics. Yvonne contributed equally with me in the cloning and characterization of RGS homologue *eat-16*, and she is always a good friend and partner. John DeModena prepared the YAC DNA for the cloning of *eat-16*. Jane Mendel taught me how to do microinjection, and developed assays we used to characterize worm locomotion. Carol Bastiani provided me the antibody for GOA-1 and paramyosin, and the plasmid construct of GPB-2. I thank the above people very much for their participation of the project. Members in Sternberg Lab helped me with stimulating discussion: Rene Garcia, Nadeem Mogal, Lorna Brundage, Chieh Chang, Byung Hwang, Aidyl Gonzalez-Serricchio…

I'd like to thank the whole *C. elegans* society for their help for the cloning of *eat-16* and *sag-4*. *C. elegans* Genetic Center (CGC) supplied us stains used for mapping and double mutants construction. Alan Coulson sent us dozens of cosmids. Yuji Kohara gave us the cDNA clones for *eat-16* and *sag-4*. M. Robozak and J. Thomas provided us *eat-16* alleles. Many people in *C. elegans* or *Drosophila* fields provided us important information.

I'd also like to thank people from Simon's group for the help in cell culture experiments. Bo Yu and Valeria Mancino taught me transfection and IP3 assay. Jennifer

Gu shared with me the Gq-G11- cell line. She and Jasen Chen helped me with lots of valuable discussions.

During the five years in Caltech, I have met quite a few people who supported me and encouraged me to overcome the hardship. My best buddy Yan Li always encourages me with a cheerful view about life. Shaoyu Wang patiently helped me to improve myself. I also thank very much Lily Yang, Jun Xie and Hongyu Ran, for the simple happiness they brought into my life as my good friends. And finally, my parents and brother in China, who always cared for me, and support me to do the best.

Abstract

In the nematode *C. elegans*, heterotrimeric G proteins have been shown to regulate the behavior of locomotion, feeding and egg-laying. Go belongs to Gi family and only exists in organisms with a nervous system. The signaling downstream of Go has been a mystery since its discovery, and it is what we are determined to find out.

Loss of Goα causes animals to be hyperactive and lay eggs constitutively. Overexpressing Goα causes opposite phenotypes. Another G protein α subunit, Gq, causes phenotypes opposite to Go. To identify G protein effectors in *C. elegans*, we performed a forward genetic screen for suppressors of activated Goα under the control of the heat-shock promoter *hsp16-2*. Because of the nature of the screen design, we identified two categories of genes. One category acts on heat shock response and the other category acts on G protein pathways. We characterized and positional cloned genes from both categories.

The second chapter of the thesis described *sag-4*, a cyclin L homologue that specifically affects heat shock promoters and decreases heat-shock induced protein expression. We propose that cyclin L is likely to be involved in heat shock induced transcription. Other genes in this category, *sag-3*, *sag-5* and *sag-8*, may also function in similar mechanisms.

The third chapter of the thesis focused on G protein signaling. *eat-16* was identified in the screen for suppressors of activated Goα. We positional cloned it and found it encodes a RGS7 homologue. RGS proteins have been studied as GTPase Activating Protein for the α subunits of heterotrimeric G proteins. Although *eat-16* was identified in a suppressor screen for activated Go (*goa-1*), both genetic and biochemical evidence showed that *eat-16* is a GAP for Gq (*egl-30*). We propose that Go and Gq antagonize each other, thereby regulating behaviors. Go might negatively regulate Gq signaling, possibly through *eat-16* or other unrevealed genes.

Chapter four describes our reconstituted system in mammalian cell culture. EAT-16 decreases Gq/G11 mediated PLC activity. GOA-1 and GPB-2 (*C. elegans* Gβ5 homologue) also decrease PLC activity induced by Gq/G11. These results are consistent

with the hypothesis that Go negatively regulate Gq signaling, and the interaction between Gβ5 and RGS7 can be one of the steps between Go and Gq.

Table of Contents

Chapter 1:

Background and introduction

Since the whole thesis is based on a screen for activated Goα under the control of a heat-shock promoter, this chapter describes background on both G protein signaling and heat-shock activated transcription. I am going to introduce heterotrimeric G proteins, RGS proteins, and signal transduction pathways related to them. Meanwhile, since part of the thesis studied heat-shock response, basal transcription mechanism and heat shock induced transcription are also reviewed.

Introduction to heterotrimeric G proteins

Heterotrimeric G proteins are a family of guanine nucleotide binding proteins broadly involved in many membrane signal transduction. The G protein heterotrimer consists of α, β and γ subunits. α is the subunit binding guanine nucleotides. It is associated with βγ subunits when inactive (bond GDP), disassociated after activation (bond GTP). β and γ always associate with each other tightly. Although there are several combinations of βγ subunits, it seems that in most of cases βγ are exchangeable among different G proteins. Individual G proteins are usually defined by α subunits because the α subunits are the most variable among G protein families. Distinct signal transduction pathways are triggered by different α subunits.

G proteins are activated by G protein Coupled Receptors (GPCR). Once the receptor is stimulated, it acts as Guanine Nucleotide Exchange Factor (GNEF) to exchange α bound GDP with GTP, which results in the disassociation of α from βγ. Both activated α and βγ subunits trigger downstream signal transduction pathways. The GTP is hydrolyzed by the intrinsic GTPase activity of α subunit, which terminates the signal, leading to the reassociation of α with βγ (Figure 1). GTPase Activating Proteins (GAP) accelerate the process of hydrolysis. One of the largest families of GAP identified for heterotrimeric G proteins is the Regulator of G Protein Signaling (RGS).

The discovery of heterotrimeric G proteins was based on a fundamental observation of the requirement of GTP when a hormone activates adenylyl cyclase (Rodbell et al., 1971). Later, It is found that G proteins are activated by receptor stimulation (Maguire et

al., 1976). Cassel and Selinger first assayed GTPase activity in vitro using turkey erythrocyte membrane (Cassel and Selinger, 1976, Cassel and Selinger, 1977, Cassel and Selinger, 1977). In the past two decades, numerous experiments were done on heterotrimeric G proteins, to study their broad functions. G protein has become one of the most important terms in modern biology.

G protein families and subfamilies

There is another large category of GTPase proteins called small G proteins, such as Ras. The small G proteins only have one polypeptide chain with about 200 amino acids. They mainly function in cell growth, differentiation, apoptosis and protein secretion. Small G proteins will not be discussed in this thesis because they function differently from heterotrimeric G proteins. The relationship between small G proteins and heterotrimeric G proteins as two major categories in the GTPase superfamily was reviewed elsewhere (Bourne et al., 1991).

Many genes have been found to encode subunits of heterotrimeric G proteins. Now there have been identified in mammals about 20 α subunits, 6 β and 8 γ. Although there are so many Gα, individual isotypes are highly conserved between species. According to the sequence diversity of the cloned α isotypes, four classes of heterotrimeric G proteins are defined (Simon et al., 1991). Each class is composed of several isotypes of Gα (Figure 2). They are named Gs, Gi, Gq and G12 subfamilies.

The Gs subfamily includes Gαs and Gαolf. Both of them stimulate adenylyl cyclase. Gαolf is expressed specifically in neuron tissues, especially olfactory epithelium. Gs and Golf are mainly activated by hormone and odorant receptors. The Gi subfamily is named after the first identified member Gi, which inhibits adenylyl cyclase. This subfamily includes Gi, Go, Gt (transducin) and Gz. Members in Gi subfamily are different from other G proteins in that they are susceptible to the modification of pertussis toxin (PTX), while Gs, Gq and G12 subfamilies are not sensitive to PTX. The modification of PTX prevents receptor-mediated activation of G proteins. The Gq subfamily includes Gq/G11,

G14 and G15/G16. Gq/G11 activate phospholipase C β pathway (Berstein et al., 1992, Gutowski et al., 1991). The G12 subfamily includes G12 and G13. They may function in regulation of channels (Voyno-Yasenetskaya et al., 1994).

A novel class of G protein, Gp, has been reported (Evans et al., 1986), which is not related to any of the four conventional subfamilies. From their low molecular weight and the low affinity with βγ, they seem to between small G proteins and the α subunits of heterotrimeric G proteins. There are arguments whether Gp should be considered as part of heterotrimeric G protein family (Gilman, 1987).

G protein signal transduction pathways

G protein subunits regulate many signal transduction pathways, including adenylyl cyclase, phospholipase C β (PLCβ) and multiple channels. Signaling from heterotrimeric G protein is carried by both α and βγ subunits. Functions of α and βγ can antagonize or enhance each other, and there may exist feed back loops from the downstream effectors of α and βγ.

Although not as much variability comparing with Gα, βγ subunits have been reported to interact directly with more than 10 kinds of proteins, such as adenylyl cyclase, PLCβ, receptors, phosducin and calmodulin. βγ were also shown as positive regulators of muscarinic K^+ channel, phospholipase A2 (PLA2), phosphoinositide 3-kinase (PI3 kinase) and β-adrenergic receptor kinase (Clapham and Neer, 1993). Recently since the discovery that Gβ5 interact with RGS proteins in vitro, more complicated issues arise on the participation of βγ on G protein regulation (Snow et al., 1998).

The function of Gα subunits depends on the individual G proteins, which also reflects the subfamily it belongs to. Gs subfamily can stimulate adenylyl cyclase and regulate Ca^{2+} channels, while Gi subfamily inhibits adenylyl cyclase (Gi), activate cGMP phosphodiesterase (transducin) and regulate Ca^{2+} and K^+ channels. Members in Gq

subfamily has been shown to activate PLC, and G12 subfamily was found to regulate Na^+/K^+ exchange (Neer, 1995).

Since heterotrimeric G proteins are composed by individual α and common βγ subunits, it is not surprising that many effectors are regulated by both α and βγ. However, it was shown that effector subtypes confer distinct patterns of regulation (Tang and Gilman, 1991). For example, PLCγ is not affected by G protein subunits, while PLCβ can be activated independently by α and βγ subunits (Smrcka and Sternweis, 1993). Certain subtypes of adenylyl cyclase are activated by α but unaffected by βγ, or activated by α but inhibited by βγ (Tang and Gilman, 1991). The complexity in the combination of α, βγ subunits and subtypes of effectors is one of the reasons that heterotrimeric G proteins are used in such a variety of pathways.

Heterotrimeric G proteins in *C. elegans*

C. elegans has become a good model system to study the in vivo function of G protein subunits. The pioneering work in isolating worm G protein homologues is done by Fino Silva et al in 1990. They identified the first *C. elegans gpa* genes, which are Gα subunits that cannot be clearly grouped into any of the four G protein subfamilies (Fino Silva and Plasterk, 1990). The same Lab also identified the first G protein β subunit in worm, which is encoded by *gpb-1* (van der Voorn et al., 1990).

The first well-studied G protein α subunit in *C. elegans* is Go, which belongs to Gi family. It is identified by screening cDNA library at low stringency using the combination of *gpa-1*, *gpa-2* and *gpa-3* as probe. *C. elegans* Goα gene *goa-1* is 81%-82% identical to mammalian homologues (Lochrie et al., 1991). *goa-1* functions in *C. elegans* locomotion, egg-laying and feeding (Mendel et al., 1995, Segalat et al., 1995). Brundage et al. studied another G protein α subunit, *egl-30*, encoding Gqα. It functions in locomotion and egg-laying, in a way opposite to *goa-1* (Brundage et al., 1996). *egl-30* is required for the viability. *C. elegans* Gsα is encoded by *gsa-1*. It is also an essential gene for the life of worms, but partial loss-of-function mutants and mosaic

overexpression (xs) lines showed defects in locomotion and egg-laying. All these three worm Gα were identified by reverse genetics. They seem to affect muscle and/or neuron activity, and defects in these genes causes abnormality in behaviors like locomotion, egg-laying and feeding.

The *C. elegans* genome-sequencing project made the process of identifying worm G proteins much easier. The essentially complete genome sequence specified a total of 20 Gα subunits, by their deduced amino acid sequences. These 20 Gα include at least one homologue from each subfamily of G proteins: *goa-1* for Gi subfamily, *egl-30* for Gq subfamily, *gsa-1* for Gs subfamily, and *gpa-12* for G12 subfamily,

Jansen et al did target deletion and expression patterns of all the G protein α subunits. According to their function, Jansen et al divided them into two groups. The first group of Gα affects neuron and/or muscle activity, such as *goa-1* (Mendel et al., 1995, Segalat et al., 1995), *egl-30* (Brundage et al., 1996) and *gsa-1* (Korswagen et al., 1997). The gene *gpa-7* also belongs to this group. *gpa-7* is most similar to Goα, but loss-of-function or overexpression of *gpa-7* causes phenotypes opposite to *goa-1* (Jansen et al., 1999). All these four genes are expressed broadly in muscle or neuron, and abnormality in them causes defective behavior in locomotion and egg-laying.

The other group of Gα defined by Jansen et al is involved in perception. Genes in this group are expressed in sensory neurons detecting chemicals (such as attractants, repellents, pheromone), nose touch, or temperature. From the expression pattern of these genes, they suggested *C. elegans* use at least 14 Gα genes in chemosensory neurons. Phenotypes corresponding to defects in chemosensation where found in mutant animals with lesions in this group of Gα subunits (Jansen et al., 1999, Zwaal et al., 1997).

There are two Gβ subunits found from *C. elegans* genome. The major Gβ subunit in worm is encoded by gene *gpb-1*, a β subunit 86% identical to mammalian Gβ1-4 (van der Voorn et al., 1990). *gpb-1* is expressed broadly on the membranes of somatic and

germline tissues. Complete loss-of-function of *gpb-1* causes lethality, however, over expressing *gpb-1* causes defects in egg laying and locomotion. Zygotic *gpb-1* is required for larval development and behaviors, while maternal *gpb-1* is needed for embryonic development (Zwaal et al., 1996). The second Gβ in *C. elegans*, encoded by *gpb-2*, is a Gβ5 homologue. Gβ5 is known for its interaction with RGS proteins (Siderovski et al., 1999), therefore studies on *gpb-2* become important given the discovery in this thesis about RGS protein EAT-16. Defects in neuronal behaviors were observed in the overexpression lines of *gpb-2* (R. Plasterk, pers. comm.)

Two Gγ subunits were found from the finished genome, *gpc-1* and *gpc-2*. Detailed study on functions or expression patterns of these two genes is in progress (Jansen et al., 1999).

G protein pathways antagonize each other to regulate certain behaviors

Several G proteins in *C. elegans* have been shown to act on the same set of behaviors. Loss of function of *goa-1* causes animals to be hyperactive and to lay eggs constitutively (Mendel et al., 1995, Segalat et al., 1995). Overexpressing *goa-1* cause opposite phenotypes in locomotion and egg-laying (Mendel et al., 1995). Another G protein α subunit, Gαq, is encoded by *egl-30*. The null allele of *egl-30* is lethal, but partial loss of function of *egl-30* causes animals to be lethargic and egg-laying defective, which is opposite to the effect of *goa-1* (Brundage et al., 1996). The worm homologue of Gαs, *gsa-1*, is also necessary for the viability of *C. elegans*. Partial loss-of-function or mosaic overexpression of *gsa-1* causes defects in egg-laying and locomotion in a way similar to *egl-30* (Korswagen et al., 1997). The major G protein β subunit in *C. elegans*, *gpb-1*, is broadly expressed, required for viability, and affects egg-laying and locomotion in a way similar to *goa-1* (Zwaal et al., 1996).

One of the most interesting characteristics in the function of these G protein subunits is that their effect on animal behavior is always dosage dependent. Two activity states of these genes correspond to opposite effects in behavior. For example, loss-of-function cause phenotypes opposite to gain-of-function and partial loss-of-function show

phenotypes less severe than null allele. This phenomenon is also shown in most genes in the same pathways of these G proteins, such as *egl-10* (RGS for *goa-1*) (Koelle and Horvitz, 1996) and *sgs-1* (adenylyl cyclase for *gsa-1*) (Korswagen et al., 1998). Their effect on behavior makes it a good approach to identify new genes in G protein signaling.

The fact that different G protein α subunits affect similar behaviors in an opposite way made us speculate that these G protein signal transduction pathways might either inhibit each other or act linearly to regulate same behavior. This is supported by observations obtained from many genetic and biochemical experiments (Hajdu-Cronin et al., 1999, Miller et al., 1996).

Regulators of G protein Signaling (RGS)

RGS is a large family of GTPase Activating Protein for Gα. The family of RGS was defined by Keolle et al in 1996 after the discovery of *sst2* from *S. cerevisiae* (Dohlman et al., 1996), and *egl-10* from *C. elegans* (Koelle and Horvitz, 1996). A novel highly conserved region in these genes was designated the RGS domain. More RGS proteins were found from many other organisms and in different isotypes (Siderovski et al., 1999). Now there exists 19 mammalian RGS (Berman and Gilman, 1998) and the *C. elegans* genome sequence has identified 12 RGS homologues (1998, Sulston et al., 1992).

Classes of RGS proteins

Not every protein containing an RGS domain functions as a regulator of G protein signaling. The mouse protein Axin has N-terminal RGS domain, but no GAP activity was reported (Zeng et al., 1997). Axin is a negative regulator of Wnt signaling in mouse embryo development and its N-terminal RGS domain is required for this function (Zeng et al., 1997). Another example is D-AKAP2, which belongs to the A-kinase Anchoring Protein family (AKAP) (Huang et al., 1997). D-AKAP2 interacts with protein kinase A (PKA) with its C-terminal. Its N-terminal has a RGS domain that lacks the residues critical for Gα interaction. These "outlier" members of RGS family do not function as classical RGS proteins, and they are not discussed in this thesis.

The classical RGS proteins can be summarized into two types according to their deduced amino acid sequences. The standard short RGS, such as RGS2 and RGS4, have short sequences containing only the RGS domain, while the long RGS, such as RGS7, RGS9 and RGS11, have a long conserved N-terminal sequence. Two domains, GGL and DEP, were defined in the N-terminal region in long RGS (Snow et al., 1998). The G protein gamma subunit-like (GGL) domain has similarity to Gγ, which suggests that RGS may interact with Gβ. The Dishevelled/EGL-10/Pleckstrin (DEP) domain is highly conserved. The DEP domain has been demonstrated to control membrane localization in Dishevelled protein (Axelrod et al., 1998). The function of DEP in RGS proteins has not been revealed.

Mechanisms of RGS function

RGS acts as a GTPase Activating Protein for Gα. It stabilizes the transition state of GTP hydrolysis to accelerate the rate of GTP hydrolysis (Berman et al., 1996, Faurobert and Hurley, 1997, Hunt et al., 1996, Watson et al., 1996). RGS proteins were found as GAPs only for Gi class and Gq class. There are crystallographic and biochemical evidence indicating that Gs class is insensitive to RGS regulation. No particular RGS was defined for G12 subfamily. However, p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13 has a N terminal domain with similarity to RGS proteins. The N-terminal domain of p115 RhoGEF activates GTPase activity of G12/G13, but not Gs, Gi. Gq subfamilies (Kozasa et al., 1998). It is worth mentioning that p115 RhoGEF is not only a GAP for Gα12/ Gα13, but also a guanine nucleotide exchange factor for Rho, a member of Ras (small G protein) superfamily. This raised the possibility that RGS proteins may have dual functions as GNEF for small G proteins and GAP for heterotrimeric G proteins.

There seems to be little specificity among RGS proteins and different Gα subunits. In the subfamily of short RGS, except RGS2 showed specificity to Gαq over Gαo (Heximer et al., 1997), most other RGS seem to be able to act on both Gq and Gi subfamily in vitro. It is more likely that the in vivo specificity of RGS to different G proteins is determined by their temporal and spatial expression patterns (reviewed by Koelle, 1996).

Strikingly in *C. elegans*, both *eat-16* and *egl-10* are RGS7 homologues (Long RGS), *egl-10* acts specifically on Go, while *eat-16* acts on Gq. Defects of these two genes cause opposite phenotypes in animal behavior (Hajdu-Cronin et al., 1999, Koelle and Horvitz, 1996). More detailed domain-swapping biochemical experiments should reveal the mechanism of specificity of these two RGS proteins.

RGS connections

Some RGS proteins (domains) were shown to interact with other proteins besides Gα. Most of the long RGS proteins have a GGL domain with similarity to Gγ, it was speculated that the GGL domain might interact with Gβ. Indeed, several groups have shown that RGS proteins can interact with Gβ5 (Siderovski et al., 1999).

Gβ5 is a Gβ subunit remotely related to Gβ1-4 (Watson et al., 1994). It does seem to be different from other Gβ subunits because none of the other Gβs interacts with RGS. RGS11 binds Gβ5 in vitro, and the binding ability is abolished by the deletion of GGL domain (Snow et al., 1998). RGS6 can also form heterodimers with Gβ5 (Snow et al., 1999). The isolation of RGS7/Gβ5 and RGS9/Gβ5L heterodimers from retinal photoreceptor cells provided in vivo evidence for the interaction (Cabrera et al., 1998). The importance of the connection between Gβ5 and RGS was demonstrated by the discovery that co-expression of Gβ5 enhances the function of RGS (Kovoor et al., 2000). Those RGS that can interact with Gβ have been classified into a new subfamily, which includes RGS6, RGS7, RGS9 and RGS11 (Siderovski et al., 1999).

The ability of RGS to interact with Gβ5 makes it more complicated to explain the G protein network. There are arguments about the effect of RGS/Gβ5 interaction. Although Snow et al observed Go GAP activity by RGS11/Gβ5, Levay et al reported that Gβ5 co-expression with RGS7 inhibits Go association. The difference between the GGL domain in RGS7 and RGS11 may explain the conflicting results. The actual mechanism RGS/Gβ5 is currently under study.

Besides Gβ5, other proteins were also reported to interact with RGS. The gene PKD1 encodes polycystin, a gene mutated in autosomal-dominant polycystic kidney disease. Kim et al reported a short-lived RGS7 degraded by ubiquitin-proteasome pathway. They found interaction between the destruction box of RGS7 and the C-terminal of polycystin. Polycystin inhibits the rapid degradation of RGS7, and membrane expression of *PKD1* causes relocalization of RGS7 (Kim et al., 1999). *C. elegans* PKD1 homologue was

identified by Barr et al in 1998, and two RGS7 homologues were reported (Hajdu-Cronin et al., 1999, Koelle and Horvitz, 1996). Genetic studies on these genes will reveal the relationship between polycystin and RGS.

RGS proteins were also reported to interact with G protein coupled receptors. RGS12 is the largest protein with a RGS box. It also has a PDZ domain (which is a protein-protein interaction motif), a phosphotyrosine-binding (PTB) domain and a GoLoco motif (which may act as receptor-independent GNEF to activate Gα). Besides being a GAP for Gi, the multidomain character of RGS12 suggests the possibility of RGS interacting with receptor, tyrosine kinase and other unknown elements in signal transduction (Siderovski et al., 1999).

RGS homologues and their functions in *C. elegans* .

There are 12 RGS homologues identified from the essentially completed *C. elegans* genome. Three of them showed function in the behavior of egg-laying (Koelle et al., 1999 pers. comm.). *egl-10* is the first RGS identified in worm (Koelle and Horvitz, 1996). Loss of function of *egl-10* causes animals to be egg-laying defective. The phenotype of *egl-10* is opposite to *goa-1*. Double mutants between *egl-10* and *goa-1* are indistinguishable from *goa-1* mutants. It indicates that *egl-10* is a negative regulator for Go. Another RGS, *eat-16*, when mutated, has phenotypes opposite to *egl-10*. Although both *egl-10* and *eat-16* are RGS7 homologues, we show here that *eat-16* act on Gq while *egl-10* act on Go (Hajdu-Cronin et al., 1999, Koelle and Horvitz, 1996). The molecular basis for the specificity is still under study.

Other genes in G protein signaling

G protein coupled Receptors

G protein coupled receptors (GPCRs) are the largest family of cell surface receptors, including all the odorant receptors. The ligands for GPCR include neurotransmitters and hormones. All GPCRs are single polypeptide chains, with seven transmembrane spans. Each transmembrane span is 20-28 amino acids long, connected to each other through hydrophilic extracellular and intracellular loops.

The most studied GPCR include adrenergic receptors, muscarinic acetylcholine receptors and serotonin receptors. The major subtypes of adrenergic receptors (α1, α2, β1 and β2) have been identified through biochemical purification followed with microsequencing /cloning or low stringency screen from cDNA library. 5-HT receptors were identified based on their pharmacological and biochemical properties, such as 5HT1A, 5HT1C and 5HT-2 receptors. There are five types of muscarinic acetylcholine receptors. M1, M3 and M5 receptors seem to couple with PLC pathways, typically mediated by Gq/G11 (Ashkenazi et al., 1989, Gutkind et al., 1991, Stephens et al., 1993), while M2 and M4 couple to adenylyl cyclase inhibition (Gutkind et al., 1991).

GPCRs are regulated in three modes (Carman and Benovic, 1998). The first is desensitization: GPCRs become less responsive after continued ligand stimulation. The process of desensitization is primarily mediated by protein kinases such as protein kinase A (PKA) and protein Kinase C (PKC). Another cause of desensitization is arrestins, which specifically bind activated GPCR and directly disrupt the receptor-G protein interaction. The second mode of regulation of GPCR is sequestration, during which receptors are physically removed from the cell surface by endocytosis. Finally, abundance of GPCR can be controlled by downregulation, which means the total cellular level of receptor decrease.

PLCβ signal transduction pathway

Downstream of heterotrimeric G protein, two major signal transduction pathways have been well studied in the past two decades. The adenylyl cyclase pathway is related to Gs and Gi, and the phospholipase C pathway is linked with Gq and βγ. The phospholipases A1 (PLA1), A2 (PLA2), C (PLC) and D (PLD) cleave phospholipids at specific sites and produce different products (Harvey et al., 1995). The products of PLC are 1, 2-diacylglycerol (DAG) and inositol 1,4,5-triphosphates (IP3), After cleavage, DAG remains at the membrane, and water-soluble IP3 goes to cytosol.

PLCβ is the subtype of PLC most commonly involved in G protein signaling. In the early 1990s, several groups purified G protein preparations from bovine brain or bovine liver. G proteins from these preparations are highly homologous to αq and α11 and they stimulate PLC activity (Smrcka and Sternweis, 1993, Stringham et al., 1992, Taylor et al., 1991). Furthur experiments demonstrated that members in Gq family interact with muscarinic receptors and mediate the stimulation of PLC by hormones. These experiments include M1 receptors activating Gq-PLC in reconstituted system (Berstein et al., 1992), and stimulation of PLCβ can be blocked by anti-Gq/G11 antibodies (Gutowski et al., 1991). These results strongly indicate that Gq subfamily is responsible for the regulation of PLC by GPCRs. After cleavage by PLCβ, Both of the products, DAG and IP3, act as secondary messengers for intracellular signal transduction. IP3 releases Ca^{2+} from intracellular Ca^{2+} pool Endoplasmic Reticulum (ER). DAG activates Protein Kinase C, the latter acts synergistically with Ca^{2+} to trigger cellular response.

DAG and Protein Kinase C

Protein kinase C is the well-known DAG receptor, which can be activated by phosphatidyleserine (PS) and DAG, in a Ca^{2+} dependent manner. PKC is also the target for phorbol ester which promotes tumor (Nishizuka, 1984).

PKC superfamily is composed of three distinct species: cPKC, nPKC and aPKC. Novel PKC (nPKC) is Ca^{2+} insensitive, activated by DAG or phorbol ester in the presence of

PS. Atypical PKC (aPKC) is Ca^{2+} insensitive and not responding to DAG/PMA (Mellor and Parker, 1998). Conventional PKC (cPKC) is activated by DAG, which increases the specificity for PS (Takai et al., 1979). cPKC is also activated by PS in a Ca^{2+} dependent way. Phorbol ester PMA targets to cPKC, activates kinase activity by eliminating the requirement for DAG (Castagna et al., 1982).

In *C. elegans*, treatment with phorbol ester causes uncoordinated movement and growth arrest (Miwa et al., 1982). In a screen for phorbol ester resistant mutants, *tpa-1* was identified and cloned as a homologue of nPKC (Tabuse et al., 1989). Another PKC gene, *pkc-1*, is identified by screening cDNA library with a PKCβ probe (Land et al., 1994). *pkc-1* also belongs to nPKC family. *C. elegans* genome sequence have identified more PKC homologues, such as *pkc-2* (*kin-11*), which belongs to cPKC (Islas-Trejo et al., 1997). Now, at least five PKC proteins have been identified, including *tpa-1, pkc-1 and pkc-2* (Sulston et al., 1992).

Heat shock induced transcription

Basal transcription mechanism in eukaryotes

RNA polymerase II (RNA pol II) mediates the transcription of all mRNAs in eukaryotes. The core enzyme of RNA pol II exists in two forms depending on its activation state. Inactivate RNA pol II (IIA) is unphosphorylated. Only after phosphorylation of its C-terminal Domain (CTD), is RNA pol II able to start transcription. Such a phosphorylated state of RNA pol II is called IIO. Many other transcription factors (TFs) are required to form a large complex with core RNA pol II enzyme. The transcription in eukaryotes can be explained by a multistep model, pre-initiation complex (PIC) assembly (Roeder, 1996). The multistep model was first realized by the purification of RNA pol II (Weil et al., 1979) from crude human cell extract, and multiple factors specific for RNA pol II was demonstrated (Matsui et al., 1980). The whole process can be explained by several steps: PIC assembly, PIC activation, initiation, elongation and termination (Roeder, 1996). A brief explanation of the process is summarized in Figure 3.

The core sequence for transcription on DNA is the promoter, including the TATA box 25-30 base pairs before the start point of transcription. Among all the basal transcription factors, TFIID is the only one with DNA binding specificity. The TATA binding subunit in TFIID is the TATA Binding Polypeptide (TBP). The first step of PIC assembly is that TBP binds specifically to TATA box. Following that, TFIIB adds to it, then preformed TFIIF-RNA polymerase IIA join the complex using TFIIB as the bridge. Sequentially the complex recruits TFIIE and TFIIH to finish PIC assembly.

PIC assembly is followed by rapid DNA melting, which is done by TFIIH. The TFIIH complex has dual functions as helicase and CTD kinase. It has two DNA helicases, XPB (yeast Ssl2) and XPD (yeast Rad3). XPB/Ssl2 unwinds DNA 3'->5', and XPD/Rad3 unwinds DNA 5'->3'. The bi-directional helicase function of TFIIH facilitates the melting of ~10bp DNA just upstream of the start site (Holstege et al., 1996). PIC at this moment is unstable. Then CTD domain of RNA pol II is phosphorylated by TFIIH, as the other part of TFIIH works as a cyclin dependent kinase. The transition of IIA to IIO form

of RNA pol II is the key to start promoter clearance: TFIIB, TFIIE and TFIIH are released from PIC complex. The left TFIIF-RNA pol IIO complex now is able to leave TBP anchor and move along DNA strand to synthesize RNA. There are other elongation factors required for the process of RNA synthesis (Reines et al., 1996). Finally when transcription terminates, RNA pol IIO is dephosphorylated. TFIIF-pol IIA is released, waiting for next round of transcription assembly.

Cyclins in transcription

Cyclins are the regulators of the cyclin dependent kinases (CDKs). The CDK kinase activity is usually regulated by phosphorylation or protein degradation of the associated cyclin. The cyclin family is defined by the existence of cyclin box (about 200 amino acids) in their protein sequence (Draetta, 1990). Highly conserved regions in the cyclin box may be critical for their structure (Furnari et al., 1997).

Originally, cyclins were discovered as cell cycle regulatory proteins. The cell cycle cyclins change their expression level depending on cell cycle stages. It was found later that some cyclins have constant expression level at all cell stages and they have functions other than controlling cell cycle (Andrews and Measday, 1998, Noble et al., 1997). One such role is the involvement of cyclin-CDK in RNA polymerase II mediated transcription.

Four types of mammalian cyclins have been found to associate with RNA pol II and regulate the phosphorylation of the C-Terminal domain (CTD) of RNA polymerase II. They are cyclin T, K, C, and H. Cyclin C associates with cdk8 (Rickert et al., 1996). There are two pairs of *S. cerevisiae* homologues of cyclin C/cdk8, *srb10/srb11* (Liao et al., 1995) and *ctk2p/ctk1p* (Sterner et al., 1995). Cyclin H associates with cdk7 (Serizawa et al., 1995). *ccl-1/kin28* are the *S. cerevisiae* homologues of cyclin H/cdk7 (Feaver et al., 1994, Valay et al., 1995). Cyclin K and cyclin T1 were also found to possess CTD kinase activity (Edwards et al., 1998, Fujinaga et al., 1998). Although the kinase partner of

cyclin K is still a mystery, cdk9 was found to be the partner for cyclin T1 (Fujinaga et al., 1998).

Identification of heat shock response

Heat shock response exists in organisms diverse from bacteria to animals and plants. It was first discovered in *Drosophila* that heat shock induces specific puffs on the polytene chromosome, indicating RNA induction and protein synthesis. Continued study led to the isolation of a series of proteins referred to as heat shock proteins (HSPs) (Lindquist, 1986, Lindquist and Craig, 1988, Nover, 1991, Peterson et al., 1979). The conserved sequences from all organisms indicate that HSPs have essential functions. Even without heat shock, most members in HSP family are expressed abundantly. The expression level elevates after heat shock.

In the past decade, heat shock proteins are revealed as molecular chaperons for protein folding, translocation, higher order assembly and degradation (Wu, 1995). During heat shock, the overexpressed HSPs protect native protein structure from damage by high temperature or other stress factors, such as heavy metal or oxidant. The function of HSPs will not be discussed in detail here. We focused on the mechanism that they are induced. The heat shock induced protein expression is one of the inducible processes commonly seen in many organisms. Studies have found that it requires a stress receptor, heat shock specific transcription factor, and elements on the promoter that interact with heat shock factors.

Heat shock induced transcription is mediated by heat shock factors.

Like most mRNA transcription, heat-shock induced mRNA synthesis is mediated by RNA polymerase II. However, it particularly requires the participation of HSE (Heat Shock responding Elements) and HSF (Heat Shock Factors) (Lis and Wu, 1993, Morimoto, 1998). The heat shock mechanism in bacteria is different from in eukaryotes. In *E. coli*, heat shock induced transcription is mediated by a specific transcription factor $\sigma 32$, which directs bacteria core RNA polymerase to heat shock promoters (Wu, 1995).

The transcription of σ32 mRNA is under the control of another heat shock induced protein, σE (Gross et al., 1990, Yura et al., 1993). In eukaryotes, heat shock induced transcription is mediated by HSF. The latter is synthesized and stored at a constant level in the cells at normal conditions (Wu, 1995). In *S. cerevisiae*, HSF trimerizes and binds HSE even without heat shock. Transcription is triggered by phosphorylation of the trimer after heat shock (Gross et al., 1990, Jakobsen and Pelham, 1991). While in other eukaryotes, HSF remains as monomer and binds non-specifically to chromatin at normal conditions, heat shock causes HSF trimerization and the trimer binds specifically to HSE with high affinity (Westwood et al., 1991).

How the binding of HSF to the HSE causes the start of transcription is still unknown. It was found that the RNA polymerase II complex docks on heat-shock promoters even without heat shock (Giardina et al., 1992, Rasmussen and Lis, 1993), so binding of HSF probably activates the release of RNA polymerase II from promoter to start transcription. It could be triggered by the cyclin-CDK phosphorylation of RNA polymerase II, as it was found that in mammalian cells after heat shock, there is a switch of unphosphorylated RNA polymerase II to the phosphorylated form (Dubois et al., 1994).

Heat shock promoters and heat shock related genes in *C. elegans*.

The heat shock proteins can be grouped into several families according to their sequence similarities: hsp70-grp78, hsp83-90, hsp60, and the low molecular weight or small HSPs (Morimoto et al., 1990). The small HSPs in Drosophila and vertebrates are about 27kd in molecular weight and they exist as large cytoplasmic aggregates (Arrigo and Ahmad-Zadeh, 1981). However, *C. elegans* small HSPs are only 16-18 kd in molecular weight. All the small HSPs from nematodes, Drosophila and vertebrates possess a region of ~72 amino acids with homology to α-crystallins (Russnak et al., 1983).

The 16 kd HSPs in *C. elegans* are encoded by four highly similar genes arranged as divergently transcribed pairs. Locus A contains *hsp16-1/hsp16-48*, and locus B contains *hsp16-2/hsp16-41*. The *hsp16-1/hsp16-48* is an inverted duplication of two divergently

transcribed genes (Figure 4) (Russnak and Candido, 1985). Although there are highly conserved structure of these genes and promoters, two hours after heat shock, locus B produces seven times more mRNA than locus A. The difference in mRNA accumulation after heat shock is probably because locus A is down regulated sooner than locus B (Jones et al., 1989). *hsp16-1* is expressed in intestine and vulva, while *hsp16-2* is expressed in neuron and muscle (Stringham et al., 1992).

Extragenic suppressors of heat shock activate Goα

Go is a member of the Gi subfamily. It is the major G protein in brain, and only exists in species with a nervous system. Go homologues have been isolated biochemically from several species, including cow (Sternweis and Robishaw, 1984, Van Meurs et al., 1987), *Drosophila* (Yoon et al., 1989), *Xenopus* (Olate et al., 1989), hamster (Hsu et al., 1990), and human (Lavu et al., 1988). In mammals, Go was found as the major growth cone protein and it is regulated by GAP-43 (Strittmatter et al., 1990). The latter is a member of growth-associated proteins tightly associated with growth cone membrane, interacting with calmodulin, IP3 kinase and PKC (Skene, 1989). In *C. elegans*, Go is expressed broadly on all the neurons and sex muscles (Mendel et al., 1995). Severe abnormality in locomotion and egg-laying behaviors are caused by loss or overexpression of *goa-1* (Mendel et al., 1995, Segalat et al., 1995). Despite the broad expression and important functions of Go, little is known about the mechanisms through which Go functions in the nervous system. The primary goal of this project is to identify downstream effectors of *goa-1*.

To identify G protein effectors in *C. elegans*, we performed a forward genetic screen for suppressors of activated Goα under the control of the *hsp16-2* promoter (Mendel et al., 1995). We obtained 24 independent alleles, falling to eight complementation groups. Because of the nature of the screen design, we identified two categories of genes, one acting on heat shock response, and the other acting on G protein pathways. We characterized and positional cloned genes from both categories.

The second chapter of the thesis will describe our works on four genes involved in heat shock induced protein expression. We focused on the cloning and analysis of *sag-4*. It encodes a *C. elegans* cyclin L, with significant identity to cyclin T, K, and C. *sag-4* decrease heat shock induced protein expression. It suppresses transgenes with the *hsp16-2* promoter, but does not affect other promoters, indicating its specificity on heat shock promoter. Cyclin T, K and C are known to function in basal transcription (Edwards et al., 1998, Fujinaga et al., 1998, Rickert et al., 1996, Serizawa et al., 1995). Based on these

results, we propose that cyclin L participate in heat shock induced transcription by functioning in TFIIH to activate cyclin dependent kinase. *sag-4* might be the missing link between the steps of HSF binding with HSE (Wu, 1995) and the phosphorylation of CTD of RNA polymerase II (Dubois et al., 1994). The other genes in this category, *sag-3*, *sag-5* and *sag-8*, may be involved in other steps during heat shock response.

The third chapter focus on G protein signaling published as Hajdu-Cronin et al, 1999. We identified two genetic loci that affect G protein signaling. *sag-1* was independently isolated and cloned as *dgk-1* (Nurrish et al., 1999). It encodes a diacylglycerol kinase, which regulates the level of DAG. The other gene, *sag-2*, is allelic to *eat-16*, which is originally isolated as a feeding mutant (Avery, 1993). Both *sag-1/dgk-1* and *eat-16* cause defects in animal behaviors in a way similar to *goa-1*. We positional cloned *eat-16* and it encodes a RGS7 homologue. Although *eat-16* was identified in a suppressor screen for activated Go (*goa-1*), we found it is not a GAP for *goa-1*. This is based on both genetic and biochemical evidence: *eat-16(lf)* and *goa-1(lf)* have similar phenotypes; *eat-16* failed to recognize *goa-1* null allele; and previous biochemical experiments indicated that RGS proteins do not act on Gα mutants with Q205->L mutation (Berman et al., 1996). We found *egl-30* is the target for *eat-16*, because of the allele-dependent suppression of *eat-16* to *egl-30* mutants, which suggested direct interaction of their gene products. Our reconstituted cell culture system also support that *eat-16* is a GAP for Gq (*egl-30*). We propose that Go and Gq antagonize each other to regulate behaviors, Go might negatively regulate Gq signaling, possibly through *eat-16* or other unrevealed genes. The model is supported by several facts. First, genes in the model have similar expression patterns, suggesting that they have chance to interact with each other (Hajdu-Cronin et al., 1999, Koelle and Horvitz, 1996, Mendel et al., 1995). Second, *dgk-1; eat-16* double mutant animals are inviable, and the viability can be restored by defects in *egl-30*. According to our model, loss of *dgk-1* and *eat-16* cause elevated DAG level and loss of *egl-30* lowers it, which explains the lethality of *dgk-1; eat-16* and the viability of *dgk-1; eat-16; egl-30* triple mutant.

The fourth chapter is about our reconstituted system in mammalian cell culture. We found *eat-16* works as a negative regulator of PLC activity induced by Gq/G11. It is consistent with the genetic hypothesis that *eat-16* is the RGS for Gq. We studied several truncation versions of EAT-16 and results indicated that GGL and RGS domains are required for its function. EAT-16 has a GGL domain and interacts with Gβ5 in vitro (C. Bastiani and P. Snow, pers. comm.). We transfected *C. elegans* Gβ5 homologue GPB-2 to the system, and found it negatively regulates Gq/G11. It suggests that GPB-2 may function together with RGS7 to regulate Gq. We also found that GOA-1 inhibits Gq/G11 induced PLC activity, indicating that Go can affect Gq signaling. It favors the linear model that Go negatively regulate Gq, possible through Gβ5 (GPB-2), DGK-1 and RGS proteins such as EAT-16.

Our genetic and cell culture reconstitution study put together two major G proteins (*goa-1* and *egl-30*) with their own RGS proteins (*egl-10* and *eat-16*). By genetic pathway analysis, we propose that Go and Gq antagonize each other to control egg-laying and locomotion behaviors. The fact that two genes in Gq signaling (*eat-16* and *dgk-1*) suppress activated Go raised the question whether Gq is downstream of Go. More biochemical assays and genetic studies will reveal the details of G protein network in *C. elegans*.

References:

(1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. The C. elegans Sequencing Consortium [published erratum appears in Science 1999 Jan 1;283(5398):35]. Science *282*, 2012-8.

Andrews, B. and Measday, V. (1998). The cyclin family of budding yeast: abundant use of a good idea. Trends Genet *14*, 66-72.

Arrigo, A.P. and Ahmad-Zadeh, C. (1981). Immunofluorescence localization of a small heat shock protein (hsp 23) in salivary gland cells of Drosophila melanogaster. Mol Gen Genet *184*, 73-9.

Ashkenazi, A., Ramachandran, J. and Capon, D.J. (1989). Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. Nature *340*, 146-50.

Avery, L. (1993). The genetics of feeding in Caenorhabditis elegans. Genetics *133*, 897-917.

Axelrod, J.D., Miller, J.R., Shulman, J.M., Moon, R.T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev *12*, 2610-22.

Berman, D.M. and Gilman, A.G. (1998). Mammalian RGS proteins: barbarians at the gate. J Biol Chem *273*, 1269-72.

Berman, D.M., Kozasa, T. and Gilman, A.G. (1996). The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. J Biol Chem *271*, 27209-12.

Berman, D.M., Wilkie, T.M. and Gilman, A.G. (1996). GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. Cell *86*, 445-52.

Berstein, G., Blank, J.L., Jhon, D.Y., Exton, J.H., Rhee, S.G. and Ross, E.M. (1992). Phospholipase C-beta 1 is a GTPase-activating protein for Gq/11, its physiologic regulator. Cell *70*, 411-8.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature *349*, 117-127.

Brundage, L., Avery, L., Katz, A., Kim, U.J., Mendel, J.E., Sternberg, P.W. and Simon, M.I. (1996). Mutations in a C. elegans Gqalpha gene disrupt movement, egg laying, and viability. Neuron *16*, 999-1009.

Cabrera, J.L., de Freitas, F., Satpaev, D.K. and Slepak, V.Z. (1998). Identification of the Gbeta5-RGS7 protein complex in the retina. Biochem Biophys Res Commun *249*, 898-902.

Carman, C.V. and Benovic, J.L. (1998). G-protein-coupled receptors: turn-ons and turn-offs. Curr Opin Neurobiol *8*, 335-44.

Cassel, D. and Selinger, Z. (1976). Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Biochim Biophys Acta *452*, 538-51.

Cassel, D. and Selinger, Z. (1977). Catecholamine-induced release of [3H]-Gpp(NH)p from turkey erythrocyte adenylate cyclase. J Cyclic Nucleotide Res *3*, 11-22.

Cassel, D. and Selinger, Z. (1977). Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. Proc Natl Acad Sci U S A *74*, 3307-11.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem *257*, 7847-51.

Clapham, D.E. and Neer, E.J. (1993). New roles for G-protein beta gamma-dimers in transmembrane signalling. Nature *365*, 403-6.

Dohlman, H.G., Song, J., Ma, D., Courchesne, W.E. and Thorner, J. (1996). Sst2, a negative regulator of pheromone signaling in the yeast Saccharomyces cerevisiae: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). Mol Cell Biol *16*, 5194-209.

Draetta, G. (1990). Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. Trends Biochem Sci *15*, 378-83.

Dubois, M.F., Bellier, S., Seo, S.J. and Bensaude, O. (1994). Phosphorylation of the RNA polymerase II largest subunit during heat shock and inhibition of transcription in HeLa cells. J Cell Physiol *158*, 417-26.

Edwards, M.C., Wong, C. and Elledge, S.J. (1998). Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity. Mol Cell Biol *18*, 4291-300.

Evans, T., Brown, M.L., Fraser, E.D. and Northup, J.K. (1986). Purification of the major GTP-binding proteins from human placental membranes. J Biol Chem *261*, 7052-9.

Faurobert, E. and Hurley, J.B. (1997). The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro. Proc Natl Acad Sci U S A *94*, 2945-50.

Feaver, W.J., Svejstrup, J.Q., Henry, N.L. and Kornberg, R.D. (1994). Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. Cell *79*, 1103-9.

Fino Silva, I. and Plasterk, R.H. (1990). Characterization of a G-protein alpha-subunit gene from the nematode Caenorhabditis elegans. J Mol Biol *215*, 483-7.

Fujinaga, K., Cujec, T.P., Peng, J., Garriga, J., Price, D.H., Grana, X. and Peterlin, B.M. (1998). The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. J Virol *72*, 7154-9.

Furnari, B.A., Russell, P. and Leatherwood, J. (1997). Pch1(+), a second essential C-type cyclin gene in Schizosaccharomyces pombe. J Biol Chem *272*, 12100-6.

Giardina, C., Perez-Riba, M. and Lis, J.T. (1992). Promoter melting and TFIID complexes on Drosophila genes in vivo. Genes Dev *6*, 2190-200.

Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. Annu Rev Biochem *56*, 615-49.

Gross, D.S., English, K.E., Collins, K.W. and Lee, S.W. (1990). Genomic footprinting of the yeast HSP82 promoter reveals marked distortion of the DNA helix and constitutive occupancy of heat shock and TATA elements. J Mol Biol *216*, 611-31.

Gutkind, J.S., Novotny, E.A., Brann, M.R. and Robbins, K.C. (1991). Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. Proc Natl Acad Sci U S A *88*, 4703-7.

Gutowski, S., Smrcka, A., Nowak, L., Wu, D.G., Simon, M. and Sternweis, P.C. (1991). Antibodies to the alpha q subfamily of guanine nucleotide-binding regulatory protein alpha subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. J Biol Chem *266*, 20519-24.

Hajdu-Cronin, Y.M., Chen, W.J., Patikoglou, G., Koelle, M.R. and Sternberg, P.W. (1999). Antagonism between G(o)alpha and G(q)alpha in Caenorhabditis elegans: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. Genes Dev *13*, 1780-93.

Heximer, S.P., Watson, N., Linder, M.E., Blumer, K.J. and Hepler, J.R. (1997). RGS2/G0S8 is a selective inhibitor of Gqalpha function. Proc Natl Acad Sci U S A *94*, 14389-93.

Holstege, F.C., van der Vliet, P.C. and Timmers, H.T. (1996). Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. Embo J *15*, 1666-77.

Hsu, W.H., Rudolph, U., Sanford, J., Bertrand, P., Olate, J., Nelson, C., Moss, L.G., Boyd, A.E., Codina, J. and Birnbaumer, L. (1990). Molecular cloning of a novel splice variant of the alpha subunit of the mammalian Go protein. J Biol Chem *265*, 11220-6.

Huang, L.J., Durick, K., Weiner, J.A., Chun, J. and Taylor, S.S. (1997). Identification of a novel protein kinase A anchoring protein that binds both type I and type II regulatory subunits. J Biol Chem *272*, 8057-64.

Hunt, T.W., Fields, T.A., Casey, P.J. and Peralta, E.G. (1996). RGS10 is a selective activator of Gαi GTPase activity. Nature *383*, 175-177.

Islas-Trejo, A., Land, M., Tcherepanova, I., Freedman, J.H. and Rubin, C.S. (1997). Structure and expression of the Caenorhabditis elegans protein kinase C2 gene. Origins and regulated expression of a family of Ca2+-activated protein kinase C isoforms. J Biol Chem *272*, 6629-40.

Jakobsen, B.K. and Pelham, H.R. (1991). A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. Embo J *10*, 369-75.

Jansen, G., Thijssen, K.L., Werner, P., van der Horst, M., Hazendonk, E. and Plasterk, R.H. (1999). The complete family of genes encoding G proteins of Caenorhabditis elegans. Nat Genet *21*, 414-9.

Jones, D., Dixon, D.K., Graham, R.W. and Candido, E.P. (1989). Differential regulation of closely related members of the hsp16 gene family in Caenorhabditis elegans. Dna *8*, 481-90.

Kim, E., Arnould, T., Sellin, L., Benzing, T., Comella, N., Kocher, O., Tsiokas, L., Sukhatme, V.P. and Walz, G. (1999). Interaction between RGS7 and polycystin. Proc Natl Acad Sci U S A *96*, 6371-6.

Koelle, M.R. and Horvitz, H.R. (1996). EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. Cell *84*, 115-25.

Korswagen, H.C., Park, J.H., Ohshima, Y. and Plasterk, R.H. (1997). An activating mutation in a Caenorhabditis elegans Gs protein induces neural degeneration. Genes Dev *11*, 1493-503.

Korswagen, H.C., van der Linden, A.M. and Plasterk, R.H. (1998). G protein hyperactivation of the Caenorhabditis elegans adenylyl cyclase SGS-1 induces neuronal degeneration. Embo J *17*, 5059-65.

Kovoor, A., Chen, C.K., He, W., Wensel, T.G., Simon, M.I. and Lester, H.A. (2000). Co-expression of Gbeta5 enhances the function of two Ggamma subunit-like domain-containing regulators of G protein signaling proteins. J Biol Chem *275*, 3397-402.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G. and Sternweis, P.C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science *280*, 2109-11.

Lackner, M.R., Nurrish, S.J. and Kaplan, J.M. (1999). Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. Neuron *24*, 335-46.

Land, M., Islas-Trejo, A. and Rubin, C.S. (1994). Origin, properties, and regulated expression of multiple mRNAs encoded by the protein kinase C1 gene of Caenorhabditis elegans. J Biol Chem *269*, 14820-7.

Lavu, S., Clark, J., Swarup, R., Matsushima, K., Paturu, K., Moss, J. and Kung, H.F. (1988). Molecular cloning and DNA sequence analysis of the human guanine nucleotide-binding protein Go alpha [published erratum appears in Biochem Biophys Res Commun 1988 May 31;153(1):487]. Biochem Biophys Res Commun *150*, 811-5.

Liao, S.M., Zhang, J., Jeffery, D.A., Koleske, A.J., Thompson, C.M., Chao, D.M., Viljoen, M., van Vuuren, H.J. and Young, R.A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature *374*, 193-6.

Lindquist, S. (1986). The heat-shock response. Annu Rev Biochem *55*, 1151-91.

Lindquist, S. and Craig, E.A. (1988). The heat-shock proteins. Annu Rev Genet *22*, 631-77.

Lis, J. and Wu, C. (1993). Protein traffic on the heat shock promoter: parking, stalling, and trucking along. Cell *74*, 1-4.

Lochrie, M.A., Mendel, J.E., Sternberg, P.W. and Simon, M.I. (1991). Homologous and unique G protein alpha subunits in the nematode Caenorhabditis elegans. Cell Regul *2*, 135-54.

Maguire, M.E., Van Arsdale, P.M. and Gilman, A.G. (1976). An agonist-specific effect of guanine nucleotides on binding to the beta adrenergic receptor. Mol Pharmacol *12*, 335-9.

Matsui, T., Segall, J., Weil, P.A. and Roeder, R.G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J Biol Chem *255*, 11992-6.

Mellor, H. and Parker, P.J. (1998). The extended protein kinase C superfamily. Biochem J *332*, 281-92.

Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin, Y.M., Simon, M.I., Plasterk, R.H. and Sternberg, P.W. (1995). Participation of the protein Go in multiple aspects of behavior in C. elegans. Science *267*, 1652-5.

Miller, K.G., Alfonso, A., Nguyen, M., Crowell, J.A., Johnson, C.D. and Rand, J.B. (1996). A genetic selection for Caenorhabditis elegans synaptic transmission mutants. Proc Natl Acad Sci U S A *93*, 12593-8.

Miwa, J., Tabuse, Y., Furusawa, M. and Yamasaki, H. (1982). Tumor promoters specifically and reversibly disturb development and behavior of Caenorhabditis elegans. J Cancer Res Clin Oncol *104*, 81-7.

Morimoto, R.I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev *12*, 3788-96.

Morimoto, R.I., Tissiers, A. and Georgopoulos, C. (1990). Stress Proteins in Biology and Medicine. Cold Spring Harbor Laboratory Press

Neer, E.J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. Cell *80*, 249-57.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature *308*, 693-8.

Noble, M.E., Endicott, J.A., Brown, N.R. and Johnson, L.N. (1997). The cyclin box fold: protein recognition in cell-cycle and transcription control. Trends Biochem Sci *22*, 482-7.

Nover, L. (1991). HSFs and HSPs--a stressful program on transcription factors and chaperones. Stress Proteins and the Heat Shock Response, sponsored by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY USA, April 29-May 2, 1991. New Biol *3*, 855-9.

Nurrish, S., Segalat, L. and Kaplan, J.M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron *24*, 231-42.

Olate, J., Jorquera, H., Purcell, P., Codina, J., Birnbaumer, L. and Allende, J.E. (1989). Molecular cloning and sequence determination of a cDNA coding for the alpha-subunit of a Go-type protein of Xenopus laevis oocytes [published erratum appears in FEBS Lett 1990 Jul 16;267(2):316]. FEBS Lett *244*, 188-92.

Peterson, N.S., Moller, G. and Mitchell, H.K. (1979). Genetic mapping of the coding regions for three heat-shock proteins in Drosophila melanogaster. Genetics *92*, 891-902.

Rasmussen, E.B. and Lis, J.T. (1993). In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc Natl Acad Sci U S A *90*, 7923-7.

Reines, D., Conaway, J.W. and Conaway, R.C. (1996). The RNA polymerase II general elongation factors. Trends Biochem Sci *21*, 351-5.

Rickert, P., Seghezzi, W., Shanahan, F., Cho, H. and Lees, E. (1996). Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene *12*, 2631-40.

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M. (1971). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanylnucleotides in glucagon action. J Biol Chem *246*, 1877-82.

Roeder, R.G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem Sci *21*, 327-35.

Russnak, R.H. and Candido, E.P. (1985). Locus encoding a family of small heat shock genes in Caenorhabditis elegans: two genes duplicated to form a 3.8-kilobase inverted repeat. Mol Cell Biol *5*, 1268-78.

Russnak, R.H., Jones, D. and Candido, E.P. (1983). Cloning and analysis of cDNA sequences coding for two 16 kilodalton heat shock proteins (hsps) in Caenorhabditis elegans: homology with the small hsps of Drosophila. Nucleic Acids Res *11*, 3187-205.

Segalat, L., Elkes, D.A. and Kaplan, J.M. (1995). Modulation of serotonin-controlled behaviors by Go in Caenorhabditis elegans. Science *267*, 1648-51.

Serizawa, H., Makela, T.P., Conaway, J.W., Conaway, R.C., Weinberg, R.A. and Young, R.A. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. Nature *374*, 280-2.

Siderovski, D.P., Strockbine, B. and Behe, C.I. (1999). Whither goest the RGS proteins? Crit Rev Biochem Mol Biol *34*, 215-51.

Simon, M.I., Strathmann, M.P. and Gautam, N. (1991). Diversity of G proteins in signal transduction. Science *252*, 802-8.

Skene, J.H. (1989). Axonal growth-associated proteins. Annu Rev Neurosci *12*, 127-56.

Smrcka, A.V. and Sternweis, P.C. (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. J Biol Chem *268*, 9667-74.

Snow, B.E., Betts, L., Mangion, J., Sondek, J. and Siderovski, D.P. (1999). Fidelity of G protein beta-subunit association by the G protein gamma-subunit-like domains of RGS6, RGS7, and RGS11. Proc Natl Acad Sci U S A *96*, 6489-94.

Snow, B.E., Krumins, A.M., Brothers, G.M., Lee, S.F., Wall, M.A., Chung, S., Mangion, J., Arya, S., Gilman, A.G. and Siderovski, D.P. (1998). A G protein gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. Proc Natl Acad Sci U S A *95*, 13307-12.

Stephens, E.V., Kalinec, G., Brann, M.R. and Gutkind, J.S. (1993). Transforming G protein-coupled receptors transduce potent mitogenic signals in NIH 3T3 cells independent on cAMP inhibition or conventional protein kinase C. Oncogene *8*, 19-26.

Sterner, D.E., Lee, J.M., Hardin, S.E. and Greenleaf, A.L. (1995). The yeast carboxyl-terminal repeat domain kinase CTDK-I is a divergent cyclin-cyclin-dependent kinase complex. Mol Cell Biol *15*, 5716-24.

Sternweis, P.C. and Robishaw, J.D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J Biol Chem *259*, 13806-13.

Stringham, E.G., Dixon, D.K., Jones, D. and Candido, E.P. (1992). Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans. Mol Biol Cell *3*, 221-33.

Strittmatter, S.M., Valenzuela, D., Kennedy, T.E., Neer, E.J. and Fishman, M.C. (1990). G0 is a major growth cone protein subject to regulation by GAP-43. Nature *344*, 836-41.

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qiu, L. and et al. (1992). The *C. elegans* genome sequencing project: a beginning. Nature *356*, 37-41.

Tabuse, Y., Nishiwaki, K. and Miwa, J. (1989). Mutations in a protein kinase C homolog confer phorbol ester resistance on Caenorhabditis elegans. Science *243*, 1713-6.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J Biol Chem *254*, 3692-5.

Tang, W.J. and Gilman, A.G. (1991). Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science *254*, 1500-3.

Taylor, S.J., Chae, H.Z., Rhee, S.G. and Exton, J.H. (1991). Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature *350*, 516-8.

Valay, J.G., Simon, M., Dubois, M.F., Bensaude, O., Facca, C. and Faye, G. (1995). The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. J Mol Biol *249*, 535-44.

van der Voorn, L., Gebbink, M., Plasterk, R.H. and Ploegh, H.L. (1990). Characterization of a G-protein beta-subunit gene from the nematode Caenorhabditis elegans. J Mol Biol *213*, 17-26.

Van Meurs, K.P., Angus, C.W., Lavu, S., Kung, H.F., Czarnecki, S.K., Moss, J. and Vaughan, M. (1987). Deduced amino acid sequence of bovine retinal Go alpha: similarities to other guanine nucleotide-binding proteins. Proc Natl Acad Sci U S A *84*, 3107-11.

Voyno-Yasenetskaya, T., Conklin, B.R., Gilbert, R.L., Hooley, R., Bourne, H.R. and Barber, D.L. (1994). G alpha 13 stimulates Na-H exchange. J Biol Chem *269*, 4721-4.

Watson, A.J., Katz, A. and Simon, M.I. (1994). A fifth member of the mammalian G-protein beta-subunit family. Expression in brain and activation of the beta 2 isotype of phospholipase C. J Biol Chem *269*, 22150-6.

Watson, N., Linder, M.E., Druey, K.M., Kehrl, J.H. and Blumer, K.J. (1996). RGS family members: GTPase-activating proteins for heterotrimeric G-proteins α-subunits. Nature *383*, 172-175.

Weil, P.A., Luse, D.S., Segall, J. and Roeder, R.G. (1979). Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA. Cell *18*, 469-84.

Westwood, J.T., Clos, J. and Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat- shock factor. Nature *353*, 822-7.

Wu, C. (1995). Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol *11*, 441-69.

Yoon, J., Shortridge, R.D., Bloomquist, B.T., Schneuwly, S., Perdew, M.H. and Pak, W.L. (1989). Molecular characterization of Drosophila gene encoding G0 alpha subunit homolog. J Biol Chem *264*, 18536-43.

Yura, T., Nagai, H. and Mori, H. (1993). Regulation of the heat-shock response in bacteria. Annu Rev Microbiol *47*, 321-50.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L., 3rd, Lee, J.J., Tilghman, S.M., Gumbiner, B.M. and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell *90*, 181-92.

Zwaal, R.R., Ahringer, J., van Luenen, H.G., Rushforth, A., Anderson, P. and Plasterk, R.H. (1996). G proteins are required for spatial orientation of early cell cleavages in C. elegans embryos. Cell *86*, 619-29.

Zwaal, R.R., Mendel, J.E., Sternberg, P.W. and Plasterk, R.H. (1997). Two neuronal G proteins are involved in chemosensation of the Caenorhabditis elegans Dauer-inducing pheromone. Genetics *145*, 715-27.

Figure legend:

Figure 1: Heterotrimeric G protein cycle.

Figure 2: G protein family tree.
(Adopted from Hepler et al., 1992.)

Sequence relationships between mammalian Gα subuunits and family groupings.

Figure 3: Sequential assembly of basal transcription complex.
(Adopted from Roeder, 1996.)

Model for pre-initiation complex (PIC) assembly and function on a TATA-containing core promoter.

Figure 4: Chromosomal structure of *hsp16* genes in *C. elegans*.
(Adopted and modified from Jones et al., 1989.)

The *hsp16-1/hsp16-41* locus (*hsp*16A) is an inverted duplication of two divergently transcribed genes. The dashed arrows indicate the transcription directions.

Figure 1

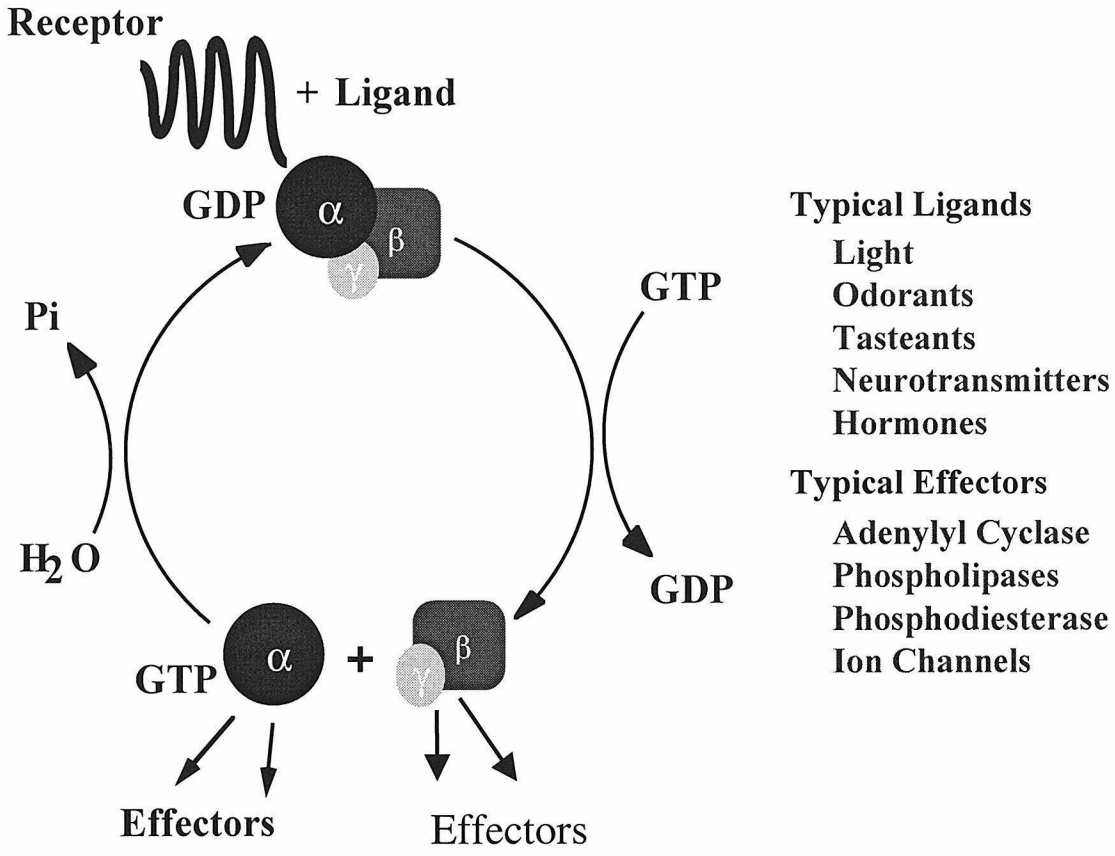

Receptor
+ Ligand
GDP
α
β
γ
GTP
Pi
H2O
GDP
GTP
α
+
β
γ
Effectors
Effectors
Typical Ligands
Light
Odorants
Tasteants
Neurotransmitters
Hormones
Typical Effectors
Adenylyl Cyclase
Phospholipases
Phosphodiesterase
Ion Channels

Figure 2

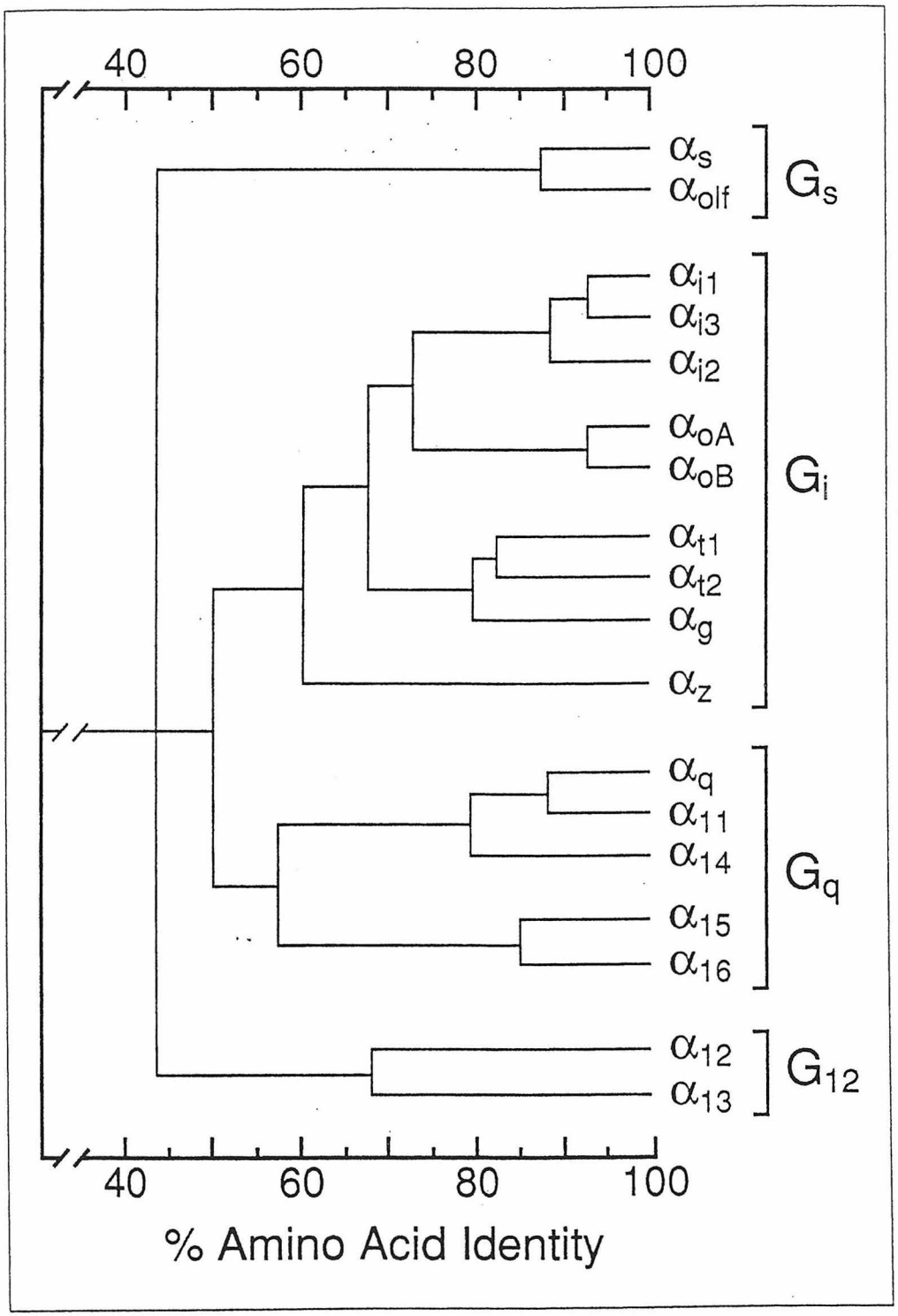

40
60
80
100
α_s
α_{olf}
G_s
α_{i1}
α_{i3}
α_{i2}
α_{oA}
α_{oB}
G_i
α_{t1}
α_{t2}
α_g
α_z
α_q
α_{11}
α_{14}
α_{15}
α_{16}
G_q
α_{12}
α_{13}
G_{12}
40
60
80
100
% Amino Acid Identity

Figure 3

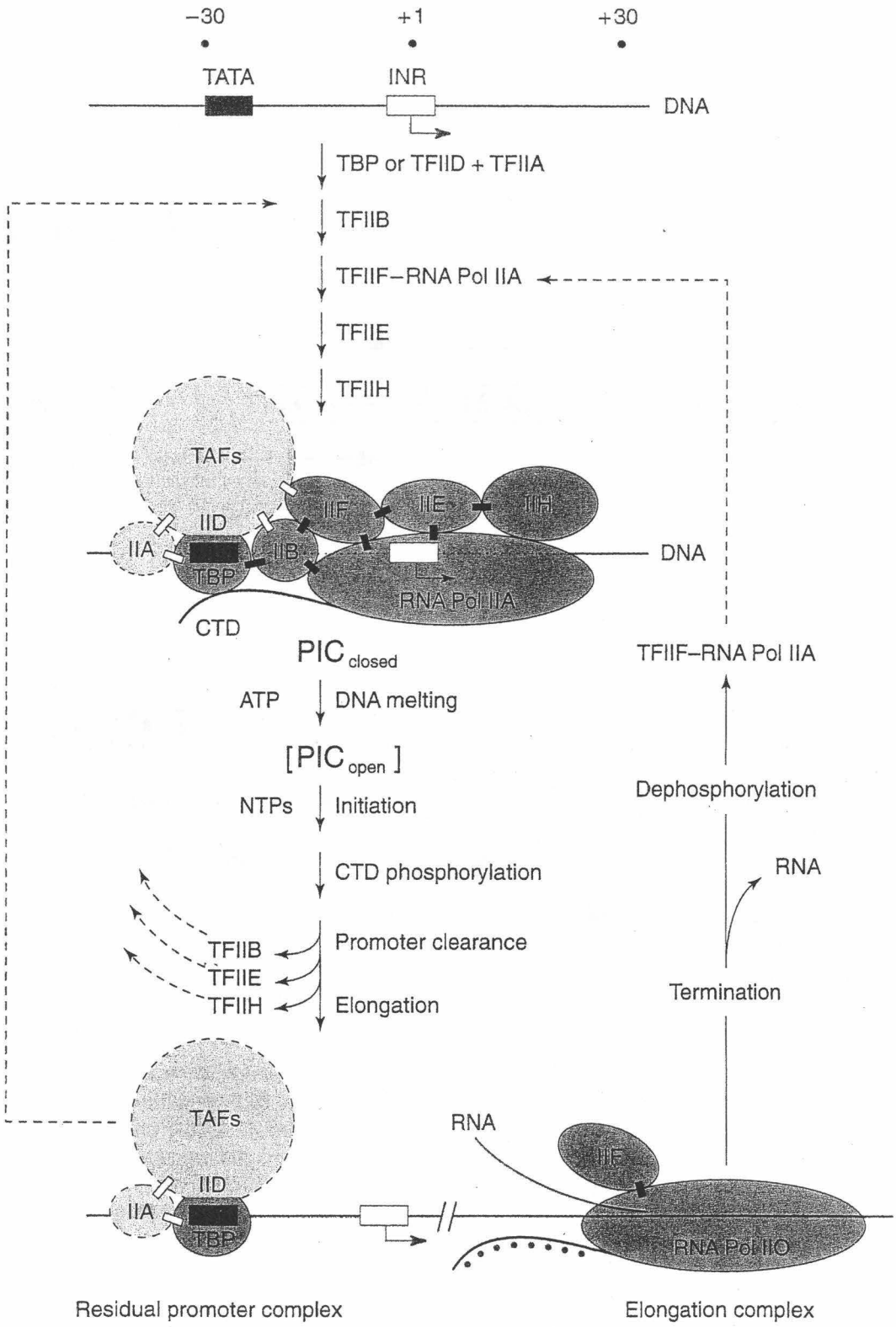
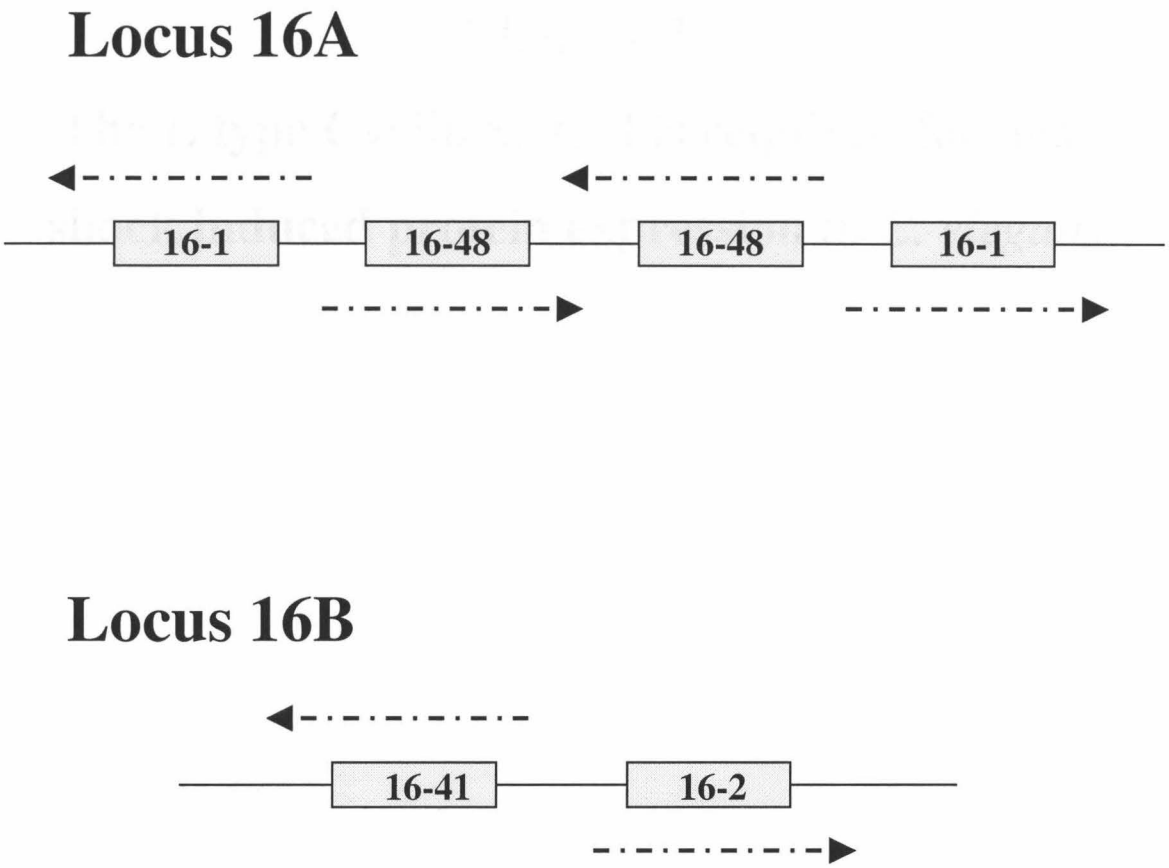

−30
+1
+30
TATA
INR
DNA
TBP or TFIID + TFIIA
TFIIB
TFIIF–RNA Pol IIA
TFIIE
TFIIH
TAFs
IID
IIA
TBP
IIB
IIF
IIE
TH
DNA
RNA Pol IIA
CTD
PIC closed
ATP
DNA melting
[PIC open]
NTPs
Initiation
CTD phosphorylation
TFIIB
TFIIE
TFIIH
Promoter clearance
Elongation
TFIIF–RNA Pol IIA
Dephosphorylation
RNA
Termination
TAFs
IID
IIA
TBP
RNA
IIF
RNA Pol IIO
Residual promoter complex
Elongation complex

Figure 4



Chapter 2:

The L type Cyclin SAG-4 is required for heat-shock induced protein expression in *C. elegans*.

Abstract:

In a screen for suppressors of activated GOA-1 under the control of a heat shock promoter, we identified four genetic loci that affect heat-shock induction of GOA-1. *sag-4* and *sag-8* are wild type in appearance, while *sag-3* and *sag-5* are egg-laying defective. Western analysis indicated that *sag-4* or *sag-8* mutations suppress activated Goα by decreasing heat-shock induced protein expression. Although endogenous GOA-1 expression is not affected, heat-shock induction of GOA-1 decreased in the suppressor strains. We cloned *sag-4* locus, which encodes a cyclin most similar to cyclin L. The latter is a novel type of cyclin with unknown function, but also similar to cyclin T, K or C, which was identified as a subunit of TFIIH, part of RNA polymerase II complex and functions in basal transcription. Only transgenes with *hsp16-2* promoter can be affected by *sag-4*. These results suggest that *sag-4* must suppress heat-shock GOA-1 phenotypes by preventing heat- shock mediated transcription in *C. elegans*. We propose that cyclin L is the type of cyclin acting in TFIIH during heat-shock induced mRNA transcription, which carries function similar to cyclin T, K or C during basal transcription. *sag-3*, *sag-5* and *sag-8* might also be involved in similar processes.

Introduction:

RNA polymerase II (pol II) is responsible for the transcription of all the mRNAs in eukaryotes. The transcription process also requires the function of other transcription factors (TFs). A multi-step model (Maldonado and Reinberg, 1995) can explain basal transcription (see Chapter I). To start transcription, the C-terminal domain of RNA polymerase II has to be phosphorylated. This critical step is mediated by TFIIH (Seroz et al., 1995). TFIIH consists of a helicase part and a cyclin-activated kinase part. The helicase subunit of TFIIH works to loosen DNA double helix, and the kinase part phosphorylates the CTD of RNA polymerase II to start the transcription (Svejstrup et al., 1996). Several types of cyclins and cyclin dependent kinases were identified to function in TFIIH. Although cyclins were originally identified in cell cycle regulatory proteins, several members of the family have been shown to be unrelated to cell cycle. Cyclin T,

K, C and H have been found to be able to regulate the phosphorylation of the C-Terminal domain (CTD) of RNA polymerase II.

In eukaryotes, heat-shock induced mRNA synthesis go through HSE (Heat Shock responding Elements) and HSF (Heat Shock Factors) (Lis and Wu, 1993, Morimoto, 1998). In eukaryotes besides *S. cerevisiae*, at normal conditions, HSF remains as monomer and its binding towards chromatin is non-specific. HSF trimerization is caused by heat shock. The trimer binds specifically to HSE with high affinity (Westwood et al., 1991). RNA polymerase II complex were found docking on heat-shock promoters without heat shock (Giardina et al., 1992, Rasmussen and Lis, 1993), and after heat shock, there is a transition of RNA polymerase II to the phosphorylated form (Dubois et al., 1994). The step between the binding of HSF to the phosphorylation of RNA pol II has been a mystery. TFIIH or other cyclin-dependent kinases are the candidates to mediate this critical process.

In this paper, we identified *sag-4*, a cyclin L homologue in *C. elegans* required for heat-shock induced transcription and might be the missing link between HSF binding and phosphorylation of RNA polymerase II. Through a genetic screen using a transgene under the control of a heat shock promoter, we isolated mutants that are likely to function during heat shock response. Roles of these genes on heat shock response are suggested by promoter specificity they showed on various transgenes, and the fact that they decrease heat shock induced protein expression, which is not correlated with the transgenes. By positional cloning of *sag-4*, analyzing its amino acids sequence and mutations sites, we believe it functions similarly to cyclin T, K, and C to phosphorylate RNA polymerase II. We propose that cyclin L is the "heat shock" cyclin that, after HSF binds HSE, acts in TFIIH to trigger the start of transcription.

Results:

Extragenic suppressors of heat shock activated Goα

Goα has been found to regulate *C. elegans* locomotion and egg-laying behaviors (Mendel et al., 1995, Segalat et al., 1995). Loss of Goα function causes animals to be hyperactive and egg-laying constitutive (wild type animals lay eggs periodically), while over-expressing of activated Goα causes opposite phenotypes. *syIs17* is an integrated transgenic line with activated Goα (Q205L) under the control of a heat-shock promoter (Mendel et al., 1995). Without heat shock, *syIs17* animals look wild type. After heat shock, animals progressively slow down in locomotion and egg-laying. About four hours after heat shock, they stop feeding, stop laying eggs, become paralyzed, and finally die. We screened *syIs17* animals mutagenized with EMS and UV-psoralen, sought for suppressors that rescue the lethality caused by heat shock. We expected to mutate genes either functioning in G protein signaling or heat-shock response. We previously described two genes identified from this screen that function in G protein signaling (Hajdu-Cronin et al., 1999). In this paper, we describe genes functioning in response to heat-shock.

From the 32,000 gametes screened (21,000 EMS and 11,000 UV-psoralen), 24 independent suppressors alleles were identified. They fall into eight complementation groups. According to their own phenotypes and mechanism they suppress *syIs17*, we classified them into five categories.

Class I include two loci, *sag-1* and *eat-16*, that when mutated, causes animals hyperactive and egg-laying constitutive. These phenotypes are similar to defects caused by other elements in G protein pathways. And indeed, they encode elements in G protein signaling (Hajdu-Cronin et al., 1999, Nurrish et al., 1999). Class I suppressors do not affect heat-shock induction of GOA-1 according to the result of western analysis (data not shown). We identified two groups of suppressors that may function in heat shock induced protein expression. Class II, composing *sag-4* and *sag-8*, are wild type in appearance. Class III composing *sag-3* and *sag*-5, are egg-laying defective (**Table 1A**).

Class II mutants suppress *syIs17* by a mechanisms different from Class I

Double mutants constructed between *sag-4* (Class II) and mutants in Class I suppress *syIs17* better than the single suppressors. *dgk-1* and *eat-16* mutations suppress *syIs17* in locomotion but not egg-laying. *sag-4* suppresses *syIs17* in both locomotion and egg laying **(Table 1B)**. The double mutant *syIs17; sag-4 (sy433); dgk-1(sy428)* moves better than *syIs17; sag-4(sy433)* or *syIs17; dgk-1(sy428)* alone (data not shown), and egg-laying is suppressed better than *syIs17; sag-4(sy433)***(Table 1B)**, A similar effect was observed in the strain *syIs17; eat-16(sy438); sag-4(sy432)* (data not shown). The different levels of suppression we observed among Class I, Class II and double mutants between Classes indicates that *sag-4* suppress *syIs17* through mechanisms different from *dgk-1* or *eat-16*. A double mutant between *sag-4* and *sag-8* may give the hint whether they function in the same pathway.

To find out how Class II mutants suppress *syIs17*, we performed western analysis to measure the heat-shock induction level of GOA-1 in suppressor strains. We found Class II suppressors decrease heat-shock induction of GOA-1 comparing with the parental strain **(Figure 1).** The endogenous GOA-1 expression was not affected by Class II suppressors (Data not shown). The results of western analysis suggest that Class II might be suppressing *syIs17* by blocking the induction of GOA-1.

***sag-4* does not function in Go signaling**

We believe that *sag-4* does not function in a normal Go pathway, since *sag-4* failed to rescue the phenotypes of *syIs9*, an integrated line of Goα promoter with activated Goα (Q205L). Strains carrying both *syIs9* and *sag-4 (sy433)* look as lethargic as *syIs9* by itself (Data not shown). The only difference between *syIs9* and *syIs17* is the promoter. We also injected the same DNA mix previously used to create stable lines of *syIs9* into *dpy-20(e1282); sag-4(sy433),* and the stable line we obtained had the same phenotype as *syIs9*. This observation indicates that *sag-4* does not suppress over-expression of activated Goα under the Go promoter. The suppression of *syIs17* can be caused by two mechanisms: *sag-4* acts specifically on the heat-shock promoter of *syIs17* as a transcription factor. Or *sag-4* acts on cells that normally do not express Goα and it only

works on the ectopically expressed Goα in *syIs17* strains. *sag-4* may regulate the over-expressed GOA-1 at transcriptional, translational or post-translational level.

We constructed a strain that overexpress *sag-4 (xs)* in the *goa-1 (n363, null)* background. *goa-1 (n363)* is hyperactive and egg-laying constitutive (Segalat et al., 1995), The overexpression (xs) stable lines of *sag-4* have wild type appearance. *sag-4(xs); goa-1(n363)* looked the same as *n363* alone. The failure of *sag-4 (xs)* to suppress *goa-1(lf)* is consistent with the result that *sag-4(rf)* failed to suppress *syIs9 (goa-1, xs)*, indicating *sag-4* does not function in normal Go signaling pathway.

We also performed western analysis to determine if endogenous expression of GOA-1 (under its own promoter) is affected by the presence of *sag-4*. There is no significant change in the levels of endogenous GOA-1 expression in *syIs17; sag-4* or *syIs17; sag-8* strains without heat-shock (Data not shown). The western result indicates that *sag-4* does not affect GOA-1 expression under control of the Go promoter, but greatly reduces GOA-1 expression under control of the heat-shock promoter. Both genetic and biochemical results suggest that *sag-4* function is heat-shock specific.

sag-4 has promoter specificity

The result of *syIs9; dpy-20(e1282); sag-4* already suggested *sag-4* is not likely a general transcriptional regulator that affects the expression of most genes in *C. elegans*. To further test if *sag-4* acts on general transcription that might non-specifically affect the expression of most reporter genes when the latter are over-expressed, we performed the following more sensitive assays to address the question **(Table 2)**.

lin-3 is an EGF homologue, which induces vulva induction in *C. elegans* (Hill and Sternberg, 1992)**.** Over-expression of *lin-3* cause animals to have a multi-vulva phenotype, and the vulva induction is highly sensitive to the expression level of *lin-3*. We used the integrated transgenic line *syIs2*, which express a *lin-3-LacZ* fusion protein under the control of the *lin-3* promoter (J. Liu, unpublished data)**.** *syIs2* itself has a multivulva

phenotype, and the strain *syIs2; unc-31; sag-4(sy433)* is phenotypically indistinguishable from *syIs2* parental strain. We conclude that the *lin-3* promoter probably is not affected by a *sag-4* mutation. By contrast, Hsieh et al reported that mutation in another *C. elegans* gene, *tam-1*, affects activity of most transgenes inlcuding those with the *lin-3* promoter (Hsieh et al., 1999).

Another promoter we tested is *let-2,* which has been used as a sensitive assay for transcription (Meneely and Wood, 1987). It encodes a α2(IV) type collagen gene in *C. elegans* (Sibley et al., 1993). There are several partial loss-of-function alleles of *let-2* very dosage sensitive to the copy number of *let-2* in the genome (Sibley et al., 1994)**.** The *let-2* (*mn114/mn114*) homozygous animals are sterile, but can survive to adulthood. However, hemizygous animals (*mn114/o*) are larval lethal. Thus, two fold reduction in *let-2* expression can be easily detected by scoring the phenotype of *let-2 (mn114); sag-4*. Double mutant *let-2 (mn114); sag-4(sy433)* survived to adulthood, indicating a *sag-4* mutation does decrease expression of *let-2*.

These double mutant results suggest that *sag-4* function does not affect every promoter, and it is possible that *sag-4* only acts on heat-shock promoter.

sag-4 suppresses other transgenes with a heat shock promoter

Since both western analysis and genetics suggest that *sag-4* may be a heat shock factor, we tested the hypothesis by constructing double mutants of *sag-4* with other transgenic lines expressing the same heat-shock promoter but other reporter genes.

syIs46 is an integrated line with heat-shock promoter controlled GFP-Lac I (A. Gonzalez-Serrichio and Sternberg, in preparation). Two hours after heat shock, GFP is expressed in the parental strain *syIs46*. While in the double mutant *syIs46; dpy-20(e1282); sag-4(sy433)*, GFP can barely be seen **(Figure 2).** Therefore *sag-4* decrease the heat shock induced GFP expression. Since GFP itself does not have functions related to G protein signaling, *sag-4* must act on the heat-shock promoter.

We also constructed a strain carry both *sag-4* mutation and *syIs38*, which is an integrated line of a heat-shock promoter with activated Gqα (C. Bastiani, unpublished data). Gqα causes phenotypes opposite to Goα in *C. elegans* egg-laying and locomotion. Two hours after heat shock, *syIs38; sag-4 (+)* animals become very hyperactive, egg-laying constitutive and have the coiled body shape. By contrast *syIs38*; *sag-4 (sy433)* animals look much less severe than *syIs38* in movement **(Figure 3)**. Suppression in the egg-laying of *syIs38* after heat shock was also observed in *syIs38*; *sag-4 (sy433)*. However, 16 hours after heat shock, both strains look hyper-contracted and no difference can be seen between them (data not shown). The partial and temporary suppression of *sag-4* to *syIs38* at 2 hours after heat shock is consistent with the hypothesis that *sag-4* act on the heat-shock promoter, thus delays the effect of heat-shock induced Gq.

The suppression of *syIs17* by *sag-4 (-)* is clean, as already indicated in Table 1B. Four hours after heat shock, the parental strain *syIs17* has become completely paralyzed, while *syIs17; sag-4 (sy433)* animals still move around, although not as well as the controls without heat shock **(Figure 3)**. The suppression is permanent, the *syIs17; sag-4* animals never become paralyzed even two days after heat shock, while *syIs17* animals never recover from the paralysis caused by heat shock. The phenotypic difference between the suppression of *sag-4* to *syIs17* and *syIs38* can be due to the difference in the level of protein expression by two transgenic lines, and the mechanism of lethality caused by two kinds of Gα.

sag-4 encodes a homologue of cyclin L

We mapped *sag-4* between *sma-1* and *lin-25* on Chromosome V by three-factor crosses. We refined *sag-4* mapping position to between *sma-1* and *egl-10*, close to *sma-1*, by deficiency mapping. 10 cosmids from the interval of *sma-1* to *egl-10* were injected to *syIs17 dpy-20; sag-4(sy433); lin-15(n765)*. Rescue of the *sag-4* phenotype was scored by failure of transgenic line to suppress *syIs17*. We found that *sag-4* was only rescued by

cosmid C52E4 but not by any of the other 9 cosmids (K12G11, R31, F09F2, F58E8, C27A7, ZK863, ZK836, K10B9 and K12F2) **(Figure 4)**.

Based on genomic sequence (Sulston et al., 1992), there are 7 Open Reading Frames (ORFs) on the cosmid C52E4. We were able to rescue *sag-4* by injecting plasmid pWJC2 containing the open reading frame C52E4.6, predicted as a weak cyclin C homologue. Another construct, pWJC3, containing the ORF C52E4.7 and the C-terminal truncated C52E4.6 did not rescue *sag-4*. We concluded that C52E4.6 corresponds to *sag-4* **(Figure 4)**.

We sequenced *sag-4* cDNA clone yk63c9 (a gift from Dr. Yuji Kohara) to determine its splicing pattern. The longest *sag-4* cDNA is 1.6 kb in length with a 140 bp 3' non-translational region. There are eight exons, the 180 amino acid cyclin box starts from exon 4 and ends in exon 7 **(Figure 4)**.

sag-4 is 39% identical to human cyclin L, 25-27% identical to human cyclin T, 24% identical to human cyclin K and 25% identical to human cyclin C. It is only18% identical to human cyclin H. In the cyclin box, the identity is higher: *sag-4* is 58% identical to human cyclin L and 30-32% identical to human cyclin T1, K and C in the cyclin box. There are four most conserved regions within cyclin box and *sag-4* is 68-68% identical to human cyclin L in these regions.

The function of human cyclin L is not yet known, while the T, K, C and H type cyclins have been implicated to function in RNA polymerase II mediated transcription. The sequence similarity among these cyclins is consistent with the hypothesis that *sag-4* act in transcription.

***sag-4* alleles are all missense mutations and they reduce *sag-4* function**

To test the hypothesis that the recessive *sag-4* alleles reduce its function, we tested their phenotypes in trans to a deficiency of the *sag-4* region. All *sag-4* alleles and *sag-4*

(*sy432*, *sy433* or *sy434*) */ctDf1* look wild type in *syIs17* background when animals are not heat-shocked. After heat shock, *syIs17/+; sag-4(sy433, sy434 or sy432)/ctDf1* behaves similarly to *syIs17; sag-4(sy433).* These observations are consistent with that all three alleles of *sag-4* reduce its function. Phenotypes of *sag-4* null mutant can be revealed by RNA interference or screening for *sag-4* deletion alleles. Over-expressing *sag-4* does not cause any phenotype in appearance or behavior (data not shown).

All three EMS-induced *sag-4* alleles have G->A mutations in genomic DNA, causing missense mutation Leu ->Phe inside the cyclin box (A.A. 109-289 in SAG-4). *sy433* has the mutation Leu158->Phe in the most conserved region 2 of cyclin box, *sy432* mutation is at Leu224->Phe in region 3, and *sy434* has Leu119->Phe in region 1 **(Figure 5)**. The mutation sites in *sy433* and *sy432* are conserved in all L, T, K and C type cyclins. However, the Leu->Phe mutation in *sy434* is only conserved in L type but not other types, which suggests that L type cyclin have its specific function different from other "transcription" cyclins.

The similar Leu->Phe mutations in *sag-4* alleles motivated us to search for Leucine zipper motif from the SAG-4 protein sequence. In the cyclin box, Repeats of Leucines or hydrophilic amino acids were found (**Figure 5B**). It is possible that mutated Leucine residues are required for the binding of SAG-4 to other proteins, such as its kinase partner. Changing the Leucines into bulky Phenyalanine residues may cause conformational changes interfering with protein interactions.

Discussion:

***sag-4* is a gene involved in heat shock response**

sag-4 was originally isolated as a suppressor of *syIs17*, an integrated transgenic line of a heat-shock promoter with activated Goα. Later we found it could also suppress other transgenic lines with the same heat-shock promoter (Mendel et al., 1995). Western analysis indicates that in *syIs17; sag-4* animals, heat-shock induced GOA-1 expression decreased approximately five folds compared to the *syIs17* parental strain, while the

endogenous GOA-1 expression is not affected. *sag-4* encodes a homologue of human cyclin L. It also has high similarity to cyclin T, cyclin K and cyclin C. All three alleles of *sag-4* we isolated have Leu -> Phe mutations in the cyclin domain, which suggests they may locate in the interface of binding and they are important for the conformation of cyclin L. Neither reduction-of-*sag-4*-function nor its overexpression causes any phenotype in the morphology and behavior of mutant animals. From the finished *C. elegans* genome, we identified independent homologues of cyclin T, K and C. The existence of independent genes encoding L, T, K, C types of cyclin in *C. elegans*, *Drosophila*, and human suggests cyclin L has specific biological functions. We propose that the L type cyclin *sag-4* is involved in RNA pol II mediated heat-shock transcription and it is required for the phosphorylation of the C terminal domain of RNA pol II after heat shock to start transcription.

Cyclin L is a novel family of cyclins

Cyclin L is a novel type of cyclin first identified from human genome (Edgar A. J., unpublished. Gene bank accession number AF180920). We have found cyclin L homologues from mouse, rat and *Drosophila* (Adams et al., 2000). The nematode *C. briggase*, a close relative to *C. elegans*, has a cyclin L homologue 75% identical to *sag-4* (1998). *The S. pombe* gene *pch1*, originally identified as a homologue of human cyclin H *and S. pombe* cyclin C, has higher similarity to L type (27% identical to human cyclin L) than to other types of cyclins. We did not find any L type cyclin from the finished *S. cerevisiae* genome. The best homologue is *srb11*, which is closer to cyclin C than to cyclin L.

sag-4 is the only cyclin L homologue we found from the almost finished *C. elegans* genomic database. The next closest homologue F44B9.2 is closer to cyclin T than to cyclin L. We have identified independent genes encoding cyclin L, T, K, and C homologues from the finished *C. elegans* genome and *Drosophila* genome **(Table 3)**. The existence of cyclin L in different species indicates it must have a specific biological function.

Cyclin L is required by heat-shock induced protein expression

Although the function of cyclin L is unknown, people have studied several types of cyclins with high similarity to cyclin L. Four kinds of mammalian cyclins (cyclin C, H, K and T1) have been shown to be part of TFIIH to assist RNA polymerase II mediated basal transcription (Edwards et al., 1998, Fujinaga et al., 1998, Rickert et al., 1996, Serizawa et al., 1995). The transcriptional cyclins, like other cyclins functioning in cell cycle, associate with their partner cyclin dependent kinases (CDK) to form active cyclin activated kinase (CAK). The function of CAK is critical, since TFIIH is required to phosphorylate the C-terminal domain of RNA polymerase II. Without cyclin, the unphosphorylated RNA polymerase II cannot be released from the promoter, and transcription fails to start **(Figure 6)**. The cyclin L homologue *sag-4* has high identity to cyclin T, K and C. It probably function similarly in transcription. The roles of cyclin T, K, C and H have been reported in the study of basal transcription. However, we found that *sag-4* only affects the heat shock promoter, but not other promoters we tested, indicating that *sag-4* has promoter specificity.

sag-4 may suppress heat-shock promoter in several ways. One possibility is that *sag-4* function is tissue specific. It is possible that *sag-4* is a general transcription factor and it is expressed only in certain tissue types, while in other tissue types, other cyclins perform similar function as *sag-4* does. In this view, *sag-4* has promoter specificity because it is expressed in the same cells as the heat-shock promoter. The heat-shock promoter we used in this paper is *hsp16-2*, mainly expressed in neurons and muscles (Stringham et al., 1992). It is possible that *sag-4* affects most of the promoters in these tissues. However, *goa-1* (Go) and *egl-30* (Gq) are also broadly expressed in neuron and muscle cells (Mendel et al., 1995) (L. Brundage and C. Bastiani pers. comm.). The fact that *sag-4* does not act on *goa-1* and *egl-30* promoters suggests that the tissue effect is unlikely the case.

Another model is that *sag-4* act specifically on heat shock promoter but not any other promoters in the same tissue. Studies have shown that during heat-shock response, HSF

(Heat Shock Factor) trimerize and bind specifically to heat shock promoter (Westwood et al., 1991). In mammalian cell culture, RNA polymerase II was found to dock on the promoter and become phosphorylated after heat shock (Dubois et al., 1994). The link between the binding of HSF and the phosphorylation of RNA polymerase II is unknown. It is likely to be mediated by TFIIH since CAK usually carry the function of phosphorylation. We propose that cyclin L is the type of cyclin for TFIIH carrying the signal from HSF to RNA polymerase II.

***sag-4* and *sag-8* may modulate, but not directly trigger heat shock response**

The other locus identified in the same screen, *sag-8*, shares similar phenotypes to *sag-4*. In *sag-8* strain, induction level of GOA-1 decreases, similar to in *sag-4* background. We believe that *sag-8* may also work on similar mechanisms as *sag-4*.

Neither gene in Class II suppresses *syIs17* back to wild type after heat shock. Residual GOA-1 expression and clear response to heat shock remains in all the Class II suppressors strains. Several possibilities can cause such a phenotype. First, it is possible that none of the suppressor alleles we obtained is null, since all the *sag-4* alleles have missense mutations, which could be the reason of incomplete suppression. Second, there may be other cyclins functionally redundant as *sag-4*. It is reported in *Drosophila* that cyclin T/cdk9 participate in heat shock transcription (Lis et al., 2000). We have identified a cyclin T homologue F44B9.2, which may have similar roles in heat shock response. Third, it is also possible that Class II suppressors act as modulators but not direct triggers of heat-shock response like heat-shock receptors or HSF. We have also got a third class (Class III) of suppressors that rescue *syIs17* back to wild type after heat shock. The phenotype of *sag-4; sag-8* double mutant will hint the relationship between these two genes.

sag-3 and *sag-5* behave differently from Class II mutants. First, they completely block heat shock induced protein expression. Second, they have defects in egg-laying behavior. Class III mutants may function differently from Class II.

Other transcription-related cyclins in *C. elegans*

There is no other gene from the worm database that showed higher similarity to human cyclin L than *sag-4*. Although there are three loci with significant identity to cyclin L (F44B9.2, F43D2.1 and H14E04.5), they were found to be more similar to other cyclin types than to L type cyclin. We identified more cyclin homologues by blasting the human cyclin T1, K and C in the finished *C. elegans* genomic database **(Table 3)**. The best worm cyclin T homologue is F44B9.2, which is 28% identical to human cyclin T1. We found a cyclin K homologue named F43D2.1, with 39% identity to human cyclin K. And there is a cyclin C homologue at cosmid H14E04.5, with 38% identity to human cyclin C. We did not find any close homologue of cyclin H during the search. The existence of L, T, K, C types of worm cyclins indicates that the L type cyclin *sag-4* probably has its specific role in *C. elegans*, possibly in heat-shock activated transcription.

Other genes in *C. elegans* that may participate in heat-shock transcription

The gene *ama-1* encodes the amanitin-binding subunit of RNA polymerase II. The hypomorph alleles of *ama-1* are temperature sensitive. They are either sterile or arrested at larval stage (Rogalski and Riddle, 1988). Several locations in *C. elegans* genome have been identified with similarity to subunits of RNA polymerase II (T06E4.8, M04G12.4, C36B1.3, F43E2.1, C42D4.8 and C26E6.4). From the finished *C. elegans* genome, we also found several genes with similarity to RNA polymerase II elongation factor (W03H1.2, F56B3.4, R12H7.5 and K08H2.1). These genes may be required for both basal and induced transcription, and they may have essential functions for the life of worms.

The only *C. elegans* HSF homologue is Y53C10A.12, with 27% identity to human HSF1. The genetic phenotypes of HSF1 and other RNA polymerase subunits have not been revealed yet, and it will be the next goal to find out by targeted deletions or RNA interference. Other genes we identified in Class II and III may encode members of them.

Though the mapping position of *sag-8* is not yet determined, *sag-3* was mapped to the right arm of Chromosome I, and *sag-5* was mapped to the left arm of chromosome III.

Transgenic lines to study elements in heat shock response

In this study we used a powerful tool to identify genes that may affect heat shock response. The integrated transgenic line *syIs17* is healthy and we can grow them like wild type before mutagenesis. After mutagenesis and heat shock, screening for mutants rescued from the lethality caused by *syIs17* is straightforward and easy to carry out. We were able to find large numbers of loci affecting heat-shock response in a relatively small-sized screen. The only problem with this method of screening for heat-shock response genes is that we also got elements affecting the G protein pathway (Class I suppressors). Designing a two step screen using the activated Goα fused with GFP can solve this. The first step is to screen for activated Goα suppressors, then examine the suppressors by epifluorescence to select for suppressors that also down regulate GFP. These suppressors should be unlikely to be involved in G protein signaling.

The mechanism of stress response has great implications in both basic research and medical applications. Although many facts have been found in mammalian cell culture about HSF and RNA polymerase II complex, there has not been a complete model to link them together. Using the powerful tool of *C. elegans* genetics, we found a potential link between HSF and RNA polymerase II complex, which allows more experiments to be performed on these genes. In vitro transcription and binding assays are necessary to test our hypothesis.

Acknowledgements:

We thank the following people and organizations: C. Bastiani for *C. elegans* GOA-1 antibody, M. Hresko for paramyosin antibody, CGC (*C. elegans* genetic center) for strains used for mapping, A. Coulson for cosmids used in positional cloning, A. Gonzalez-Serricchio and C. Bastiani for strains used for double mutant construction and help in characterizing the phenotypes, Y. Hajdu-Cronin, B. Hwang, R. Garcia, C. Chang,

C. Bastiani and other members our laboratory for helpful discussions. Most mapping information was obtained from the *C. elegans* database ACeDB.

Methods and materials:

Standard procedure (Brenner, 1974) was used to for *C. elegans* culture. Nematodes were grown and characterized at 20 °C unless indicated. The following strains were used for genetic mapping and double mutant construction. LGI: *syIs17 dpy-20(e1282); eat-16(sy438)*, *syIs17 dpy-20(e1282); goa-1(n363)*. LGIV: *syIs17 dpy-20(e1282)* (Mendel et al., 1995), *syIs2; unc-31(e169)* (J. Liu, pers. comm.), *syIs38; dpy-20(e1282)* (C. Bastiani, pers. comm.), *syIs46; ncl-1(e1865); dpy-20(e1282); him-5(e1490)*, *syIs9; dpy-20(e1362)* (Mendel et al., 1995) LGV: *syIs17 dpy-20(e1282); dpy-11(e224) unc-42(e270) him-5(e1490)*, *syIs17 dpy-20(e1282); unc-42(e270) lin-25(sy29)*, *syIs17 dpy-20(e1282); sma-1(e30) lin25(sy29)*, *syIs17 dpy-20(e1282); unc-420(e270) lin-25(sy29)*, *+/ DnT1 [unc(n754)let]; ctDf1/ DnT1 [unc(n754)let]*, *+/ letnT1; unc-42(e270) arDf1/ letnT1*. LGX: *syIs17 dpy-20(e1282); dgk-1(sy428)*, *syIs17 dpy-20(e1282); lin-15(n765)*; *mnDp1(X;V)/ +; unc-3(e151) let-2(mn114)*.

Screen and characterization of suppressors

The genetic screen has already been described (Hajdu-Cronin et al., 1999). All suppressor strains were back crossed at least three times to the parental strain *syIs17 dpy-20(e1282)*. The suppression of the lethality induced by *syIs17* was used as the criterion to follow the suppressors during all the genetic crosses.

To score egg-laying-rate /hour. L4 animals were picked the day before characterization. Heat shock at 33 °C for 30 minutes was performed 24 hour after mid-L4. After heat shock, animals were incubated at 20 °C for 10 hours. Egg-laying-rate/hour was tested by placing one animal on each two-day-old bacterial OP50 and letting lay eggs for 4 hours at 20 °C.

Genetic mapping of *sag-4*

sag-4 was mapped between *unc-42 and lin-25* by selecting Unc-non-Egl or Egl-non-Unc recombinants from *unc-42 + lin25/+ sag-4 +* heterzygots; 1 out of 3 Unc-non-Egl recombinants picked up *sag-4* and 5 out of 6 Egl-non-Unc recombinants picked up *sag-4*. *sag-4* was mapped to the right of *sma-1* by selecting Unc-non-Sma or Sma-non-Unc recombinants from *sag-4/unc-42 sma-1*; all 11 Unc-non-Sma recombinants picked up *sag-4*, and none of the 8 Sma-non-Unc recombinants picked up *sag-4*. These data were confirmed by three factor mapping of *sag-4* with *sma-1 lin-25* by picking Egl-non-Sma recombinants from *sma-1 + lin-25/+ sag-4 +*, 6 out of 7 recombinants picked up *sag-4*.

To localize *sag-4* position within the interval of *sma-1* and *lin-25*, we did deficiency mapping with *ctDf1* and *arDf1*. *sag-4* failed to complement *ctDf1 (syIs17/+; ctDf1/sag-4(sy433)*, n>50). *ctDf1* was previously mapped between *daf-11* and *egl-10* (*ctDf1* does not delete either of these two genes, (Manser and Wood, 1990), locating *sag-4* between *sma-1* and *egl-10*. Consistent with the above results, *sag-4* complements *arDf1* (*syIs17/+; sag-4(sy433)/arDf1*, n=17). The left end of *arDf1* was mapped close to *sma-1* (*arDf1* does not delete *sma-1* (Tuck and Greenwald, 1995).

The three-factor mapping and deficiency assay suggested *sag-4* is located to the right of *sma-1* and left of *egl-10*, close to *sma-1*.

Transformation rescue of *sag-4*

10 cosmids in the interval of *osm-6* and *egl-10* was selected and purified with Qiagen cosmid prep protocol. Cosmids were then paired in two as a pool and microinjected (Mello and Fire, 1995) to *syIs17 dpy-20(e1282); sag-4(sy433); lin-15(n765)* at the concentration of 50ng/µl for each cosmid, 50ng/µl of *lin-15* genomic DNA pbLH98 Total DNA concentration was normalized to 200ng/µl with pBluescript. Once rescue is confirmed the cosmids from the pool were then injected one by one to for the positive clone. The injection strain was grown at 15 °C before injection, after injection the animals were cultured at 22 °C to score the temperature sensitive *lin-15* Muv phenotype. F1 non-Muv animals were selected 4 to 5 days after injection. In the next generation stable lines

were selected from the F1 transformants. More than 3 stable lines were required to confirm the rescue status for each cosmid. Four stable lines were obtained from the injection of cosmid C52E4, all the stable lines showed 40% to 70% rescue. The cosmid F09F2 has about 70% overlap with the left end of C52E4 but it does not rescue *sag-4*, thus indicated *sag-4* was located at the right end of C52E4.

A 6.6kb BamH1/Asp718 fragment from C52E4 containing the last two open reading frames was subcloned into pBluscript (pWJC1). All 6 stable lines obtained from injected pWJC1 (43ng/μl) showed >90% rescue. A 3.8kb Asp718/SacI fragment from pWJC1 containing C52E4.6 was subcloned into pBluscript (pWJC2). Injection of pWJC2 gave us 6 stable lines at 10ng/μl and 2 stable lines from 30ng/μl), *sag-4* were rescued in all of them. Another subclone of a 4.0kb BamH1/Xho1 fragment from pWJC1 containing C52E4.7 and the C-terminal truncated C52E4.6 (pWJC3) did not rescue *sag-4* (1 stable lines at 10ng/μl, and 3 stable lines at 30ng/μl).

Sequencing *sag-4* mutation alleles

A 3kb genomic DNA fragment was amplified (Williams et al., 1992) from 8-10 L1 to L2 stage animals of *sag-4 (sy433)* using Expand long-range PCR kit (Boehringer Mannheim) with primer S4P6 (5' to 3': TACGTGACGGTGTACCGTCAAAG) and primer S4P10(5' to 3': AACAGAACCGTGCTTGCGGAAC). The primary PCR product was gel purified and used as template for nest PCR using primers S4P10 and S4P13 (5' to 3': CGGCAACCGCTACGCAG). And the secondary PCR product was gel purified and directly sequenced. Mutation site was obtained by comparing sequence from PCR fragment to wild type genomic sequence from GenBank. The point mutation identified from this sample was later confirmed by directly sequencing three independent primary PCR samples using S4P6 and S4P10. The *sy432* and *sy434* alleles were sequenced as above except the secondary PCR primers are S4P6 and S4P10.

***sag-4* cDNA sequencing**

Full-length cDNA sequence of *sag-4* was obtained from clone yk63c9, a gift from Dr. Yuji Kohara. The λZapII clones were excised in vitro and amplified in SOLR cells (Maniatis et al., 1982). Purified phagemids were then sequenced using primers for T3, T7 promoters and primers used for allele sequencing. The splicing pattern was obtained by comparing yk63c9 with wild type *sag-4* genomic sequence obtained from GenBank.

Double mutant construction

Since *sag-4* does not have a phenotype of its own, we used a balancer mutation to cross *sag-4* out of the *syIs17* background. The double mutants with *syIs46*, *syIs2* and *let-2* were made by crossing *syIs17; sag-4* to these strains and heat shock at the F2 generation to select *sag-4* homozygots in the background of *syIs17*/+. The other mutation (*syIs46*, *syIs2* or *let-2*) was homozygosed, and finally *syIs17* was eliminated from the background by subcloning animals that did not respond to heat shock.

syIs9 and *syIs38* have phenotypes interfering with the scoring of *syIs17* and they all use the same injection marker *dpy-20*, so these strains were first crossed to *dpy-20; unc-42* to use *unc-42* to balance *sag-4* later. *dpy-20; sag-4* males were crossed to *syIs9* or *syIs38; dpy-20; unc-42*. *sag-4* mutation was followed by selecting homozygous non-Unc. *syIs9* and *syIs38* transgenes were followed by selecting non-Dpy animals.

The double mutant *syIs17; goa-1(n363); lin-15; syEx240(sag-4 xs)* was constructed by crossing *syIs17; lin-15; syEx240* to *syIs17; goa-1(n363); lin-15*; *syEx240* was followed by selecting non-Muv animals.

References:

(1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. The C. elegans Sequencing Consortium [published errata appear in Science 1999 Jan 1;283(5398):35 and 1999 Mar 26;283(5410):2103 and 1999 Sep 3;285(5433):1493]. Science *282*, 2012-8.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., George, R.A., Lewis, S.E., Richards, S., Ashburner, M., Henderson, S.N., Sutton, G.G., Wortman, J.R., Yandell, M.D., Zhang, Q., Chen, L.X., Brandon, R.C., Rogers, Y.H., Blazej, R.G., Champe, M., Pfeiffer, B.D., Wan, K.H., Doyle, C., Baxter, E.G., Helt, G., Nelson, C.R., Gabor Miklos, G.L., Abril, J.F., Agbayani, A., An, H.J., Andrews-Pfannkoch, C., Baldwin, D., Ballew, R.M., Basu, A., Baxendale, J., Bayraktaroglu, L., Beasley, E.M., Beeson, K.Y., Benos, P.V., Berman, B.P., Bhandari, D., Bolshakov, S., Borkova, D., Botchan, M.R., Bouck, J., Brokstein, P., Brottier, P., Burtis, K.C., Busam, D.A., Butler, H., Cadieu, E., Center, A., Chandra, I., Cherry, J.M., Cawley, S., Dahlke, C., Davenport, L.B., Davies, P., de Pablos, B., Delcher, A., Deng, Z., Mays, A.D., Dew, I., Dietz, S.M., Dodson, K., Doup, L.E., Downes, M., Dugan-Rocha, S., Dunkov, B.C., Dunn, P., Durbin, K.J., Evangelista, C.C., Ferraz, C., Ferriera, S., Fleischmann, W., Fosler, C., Gabrielian, A.E., Garg, N.S., Gelbart, W.M., Glasser, K., Glodek, A., Gong, F., Gorrell, J.H., Gu, Z., Guan, P., Harris, M., Harris, N.L., Harvey, D., Heiman, T.J., Hernandez, J.R., Houck, J., Hostin, D., Houston, K.A., Howland, T.J., Wei, M.H., Ibegwam, C., et al. (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185-95.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Dubois, M.F., Bellier, S., Seo, S.J. and Bensaude, O. (1994). Phosphorylation of the RNA polymerase II largest subunit during heat shock and inhibition of transcription in HeLa cells. J Cell Physiol *158*, 417-26.

Edwards, M.C., Wong, C. and Elledge, S.J. (1998). Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity. Mol Cell Biol *18*, 4291-300.

Fujinaga, K., Cujec, T.P., Peng, J., Garriga, J., Price, D.H., Grana, X. and Peterlin, B.M. (1998). The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. J Virol *72*, 7154-9.

Giardina, C., Perez-Riba, M. and Lis, J.T. (1992). Promoter melting and TFIID complexes on Drosophila genes in vivo. Genes Dev *6*, 2190-200.

Hajdu-Cronin, Y.M., Chen, W.J., Patikoglou, G., Koelle, M.R. and Sternberg, P.W. (1999). Antagonism between G(o)alpha and G(q)alpha in Caenorhabditis elegans: the

RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. Genes Dev *13*, 1780-93.

Hill, R.J. and Sternberg, P.W. (1992). The gene lin-3 encodes an inductive signal for vulval development in C. elegans [see comments]. Nature *358*, 470-6.

Hsieh, J., Liu, J., Kostas, S.A., Chang, C., Sternberg, P.W. and Fire, A. (1999). The RING finger/B-box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in Caenorhabditis elegans. Genes Dev *13*, 2958-70.

Lis, J. and Wu, C. (1993). Protein traffic on the heat shock promoter: parking, stalling, and trucking along. Cell *74*, 1-4.

Lis, J.T., Mason, P., Peng, J., Price, D.H. and Werner, J. (2000). P-TEFb kinase recruitment and function at heat shock loci [In Process Citation]. Genes Dev *14*, 792-803.

Maldonado, E. and Reinberg, D. (1995). News on initiation and elongation of transcription by RNA polymerase II. Curr Opin Cell Biol *7*, 352-61.

Manser, J. and Wood, W.B. (1990). Mutations affecting embryonic cell migrations in Caenorhabditis elegans. Dev Genet *11*, 49-64.

Mello, C. and Fire, A. (1995). DNA transformation. Methods Cell Biol *48*, 451-82.

Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin, Y.M., Simon, M.I., Plasterk, R.H. and Sternberg, P.W. (1995). Participation of the protein Go in multiple aspects of behavior in C. elegans [see comments]. Science *267*, 1652-5.

Meneely, P.M. and Wood, W.B. (1987). Genetic analysis of X-chromosome dosage compensation in Caenorhabditis elegans. Genetics *117*, 25-41.

Morimoto, R.I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev *12*, 3788-96.

Nurrish, S., Segalat, L. and Kaplan, J.M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron *24*, 231-42.

Rasmussen, E.B. and Lis, J.T. (1993). In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc Natl Acad Sci U S A *90*, 7923-7.

Rickert, P., Seghezzi, W., Shanahan, F., Cho, H. and Lees, E. (1996). Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene *12*, 2631-40.

Rogalski, T.M. and Riddle, D.L. (1988). A Caenorhabditis elegans RNA polymerase II gene, ama-1 IV, and nearby essential genes. Genetics *118*, 61-74.

Segalat, L., Elkes, D.A. and Kaplan, J.M. (1995). Modulation of serotonin-controlled behaviors by Go in Caenorhabditis elegans [see comments]. Science *267*, 1648-51.

Serizawa, H., Makela, T.P., Conaway, J.W., Conaway, R.C., Weinberg, R.A. and Young, R.A. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. Nature *374*, 280-2.

Seroz, T., Hwang, J.R., Moncollin, V. and Egly, J.M. (1995). TFIIH: a link between transcription, DNA repair and cell cycle regulation. Curr Opin Genet Dev *5*, 217-21.

Sibley, M.H., Graham, P.L., von Mende, N. and Kramer, J.M. (1994). Mutations in the alpha 2(IV) basement membrane collagen gene of Caenorhabditis elegans produce phenotypes of differing severities. Embo J *13*, 3278-85.

Sibley, M.H., Johnson, J.J., Mello, C.C. and Kramer, J.M. (1993). Genetic identification, sequence, and alternative splicing of the Caenorhabditis elegans alpha 2(IV) collagen gene. J Cell Biol *123*, 255-64.

Stringham, E.G., Dixon, D.K., Jones, D. and Candido, E.P. (1992). Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans. Mol Biol Cell *3*, 221-33.

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qiu, L. and et al. (1992). The C. elegans genome sequencing project: a beginning [see comments]. Nature *356*, 37-41.

Svejstrup, J.Q., Vichi, P. and Egly, J.M. (1996). The multiple roles of transcription/repair factor TFIIH. Trends Biochem Sci *21*, 346-50.

Tuck, S. and Greenwald, I. (1995). lin-25, a gene required for vulval induction in Caenorhabditis elegans. Genes Dev *9*, 341-57.

Westwood, J.T., Clos, J. and Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat- shock factor. Nature *353*, 822-7.

Williams, W.V., Sato, A., Rossman, M., Fang, Q. and Weiner, D.B. (1992). Specific DNA amplification utilizing the polymerase chain reaction and random oligonucleotide primers: application to the analysis of antigen receptor variable regions. DNA Cell Biol *11*, 707-20.

Figure legend:

Figure 1: Class II and III mutants suppress *syIs17* by reducing the expression of heat-shock activated GOA-1 expression.
Top: In *syIs17* background, both loci we identified in Class II down regulate the heat-shock-GOA-1 induction. Bottom: In a *syIs17; goa-1(n363)* background both loci in Class III down regulate heat-shock induction of GOA-1

Figure 2: Suppression of heat shock induced GFP (*syIs46*) by *sag-4*.
syIs46 is the transgenic line with promoter hsp16-2 controlled GFP. The expression of GFP is not affected by a *ncl-1* or *him-5* mutation (data not shown). Photos were taken 2 hours after heat shock under epifluorescence with a 100X Neoflurescent object lens. Top: GFP expression in the strain *syIs46; ncl-1; him-5(e1490).* Bottom: GFP expression in the strain *syIs46; ncl-1; sag-4(sy433).*

Figure 3: Suppression of *sag-4* to *syIs17* (*hsp16-2::GoQL*) and suppression of *sag-4* to *syIs38* (*hsp16-2::*GoQL). Each photo shows the track of 5 animals for 5 minutes on the bacterial lawn with or without heat shock. Photos were taken at magnification 125X. A. *syIs17* without heat shock. B: *syIs17; sag-4(sy433)* without heat shock. C: *syIs17* 4 hours after heat shock. D: *syIs17; sag-4(sy433)* 4 hours after heat shock. E: *syIs38* without heat shock. F: *syIs38; sag-4(sy433)* 2 hours after heat shock. G: *syIs38* 2 hours after heat shock. H: *syIs38; sag-4(sy433)* 2 hours after heat shock.

Figure 4: Cloning of *sag-4*.
A. Genetic mapping and positional cloning of *sag-4*. *sag-4* is mapped between *sma-1* and *lin-25* by three-factor mapping, then between *sma-1* and *egl-10* by deficiency mapping. 10 cosmids during the interval was injected and rescue was only obtained by C52E4. B. Subcloning of cosmid C52E4 to identify *sag-4* open-reading-frame. Rescuing plasmid pWJC1 contains both C52E4.6 and C52E4.7. Rescuing plasmid pWJC2 contains C52E4.6. The plasmid pWJC3 contains C52E4.7 did not rescue *sag-4*. C: Splicing pattern of *sag-4*. *sag-4* has 8 exons and 7 introns. Cyclin box extends from exon4 to exon7.

Figure 5: Sequence alignment of *sag-4* with human cyclins.
A. Shown is the total length of the whole protein and the location and length of cyclin box in each cyclin homologue. B. Shown is identity of *sag-4* with human cyclins in the cyclin box. Four conservative regions in cyclin box are marked. Dark area indicates identical region. Arrowheads mark*sag-4* mutation sites, all of them are Leu->Phe mutations.

Figure 6: Model for *sag-4* functioning in heat-shock induced transcription.

Table 1: Class I and Class II suppressors of *syIs17*.
A. Shown in the top panel: *dgk-1* and *eat-16* as Class I suppressors. Bottom panel: *sag-4* and *sag-8* as class II suppressors. All the alleles of *sag-4* are obtained from EMS mutagenesis. *sag-8(sy530)* is obtained from UV-psoralen mutagenesis. B: Double mutants between Class I and II suppress *syIs17* better than the single suppressors. Egg-laying assay were done 10-14 hours after heat shock at 20 degree. Egg-laying rate per hour from each animal was calculated as the average of 4 hours. For each condition, average and standard deviation was calculated upon 10 animals. To determine if the difference between the strains is significant, data were calculated using the software INSTAT.

Table 2: Promoter specificity of *sag-4*.
Double mutants between *sag-4* and various strains with specific promoter and reporter genes were constructed. Assays were done according to the genetic phenotype of each reporter gene to determine whether *sag-4* affects the expression. All reporter genes controlled by heat-shock promoter were suppressed. None of the reporter genes controlled by other promoters was affected by *sag-4*.

Table 3: Cyclin L, T, K and C from *C. elegans*, *Drosophila* and Human.

Sequences from *C. elegans*, *Drosophila* and Human genomes were identified by basic blast search. Shown in the table is the percentage identity between genes. Same cyclin types have highest identity (Bold), while different cyclin types have lower identity.

Figure 1

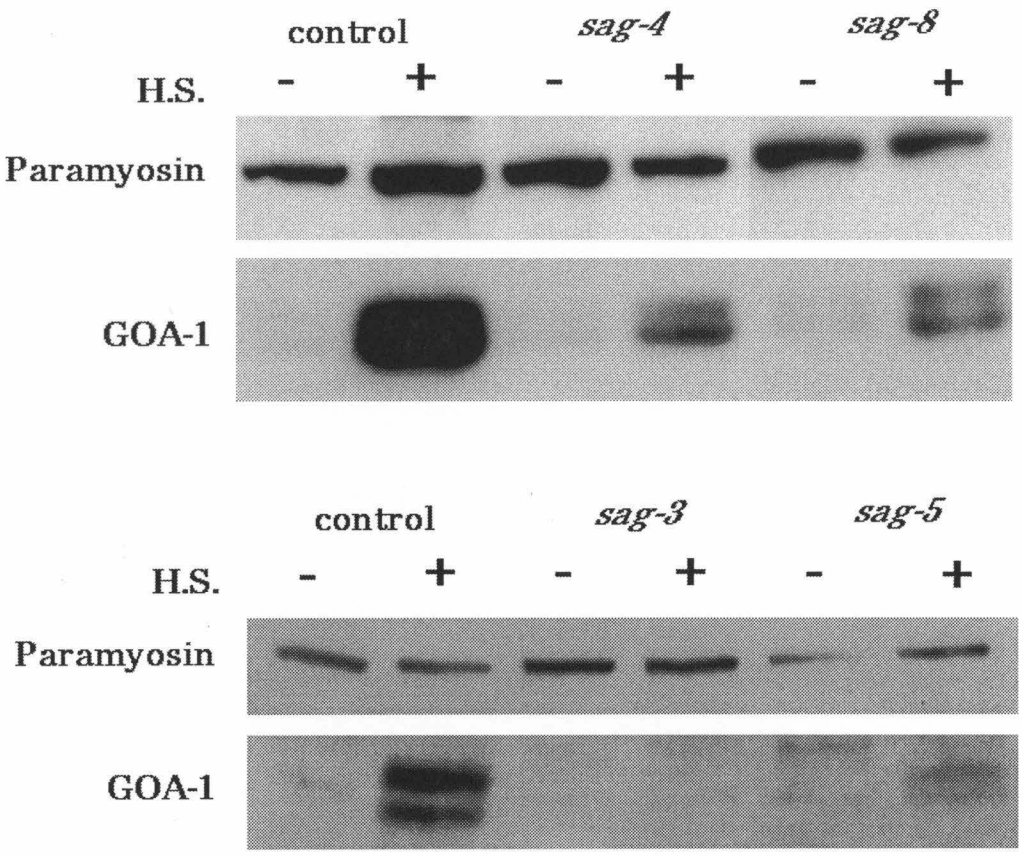

control
sag-4
sag-8
H.S.
- + - + - +
Paramyosin
GOA-1
control
sag-3
sag-5
H.S.
- + - + - +
Paramyosin
GOA-1

Figure 2

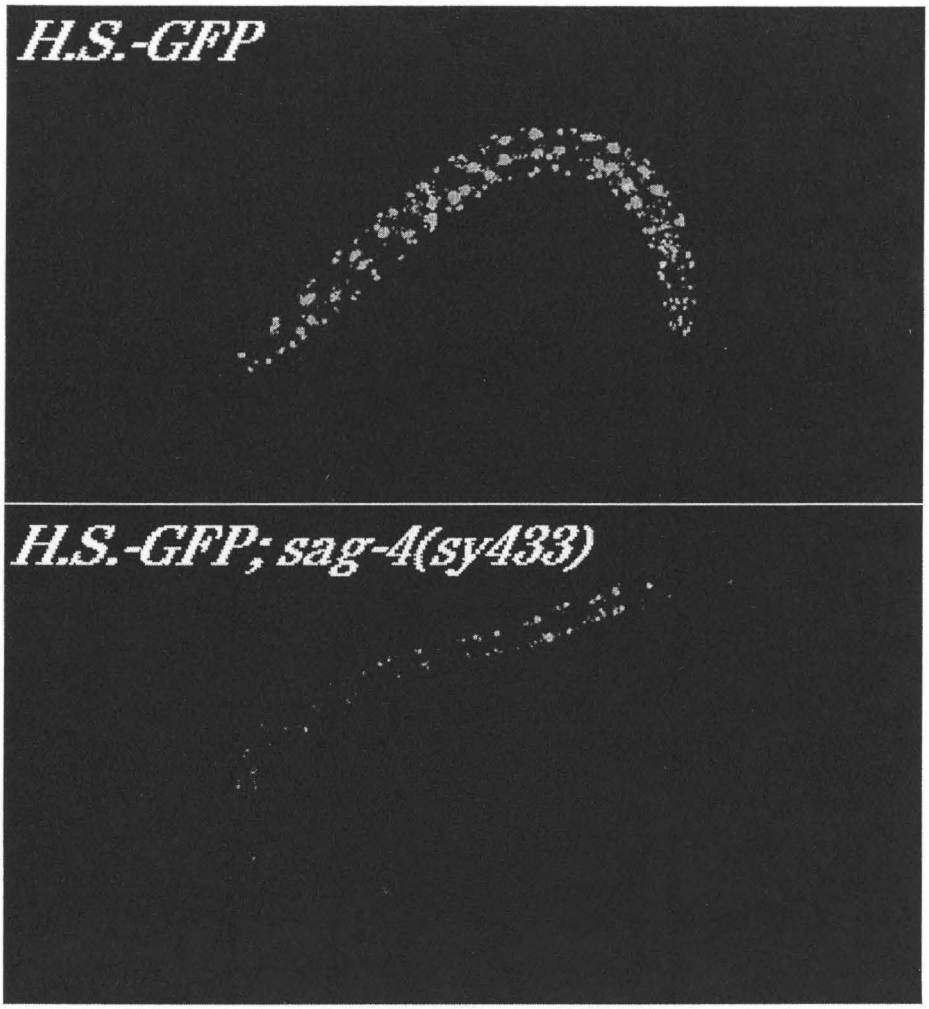


Figure 3

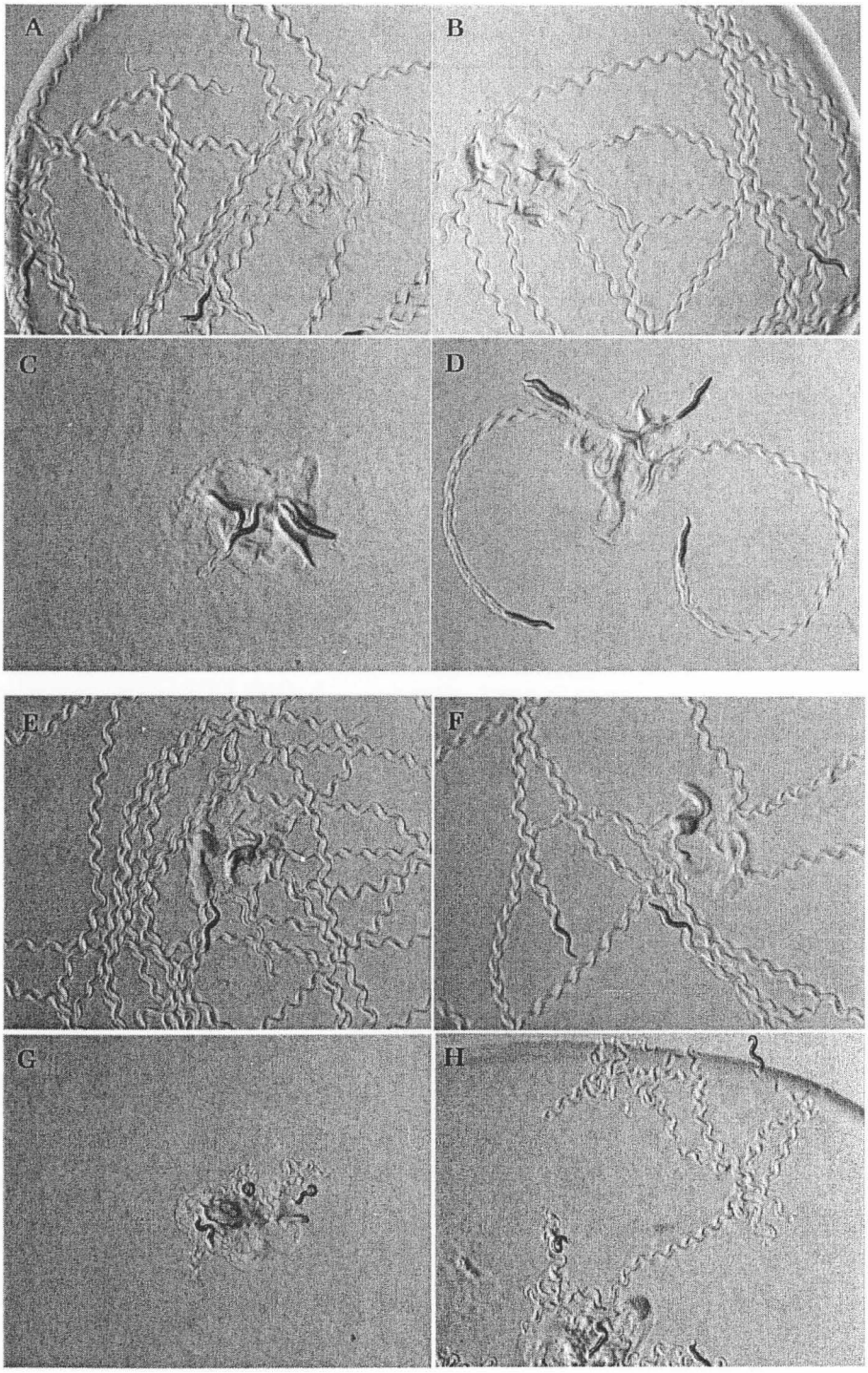
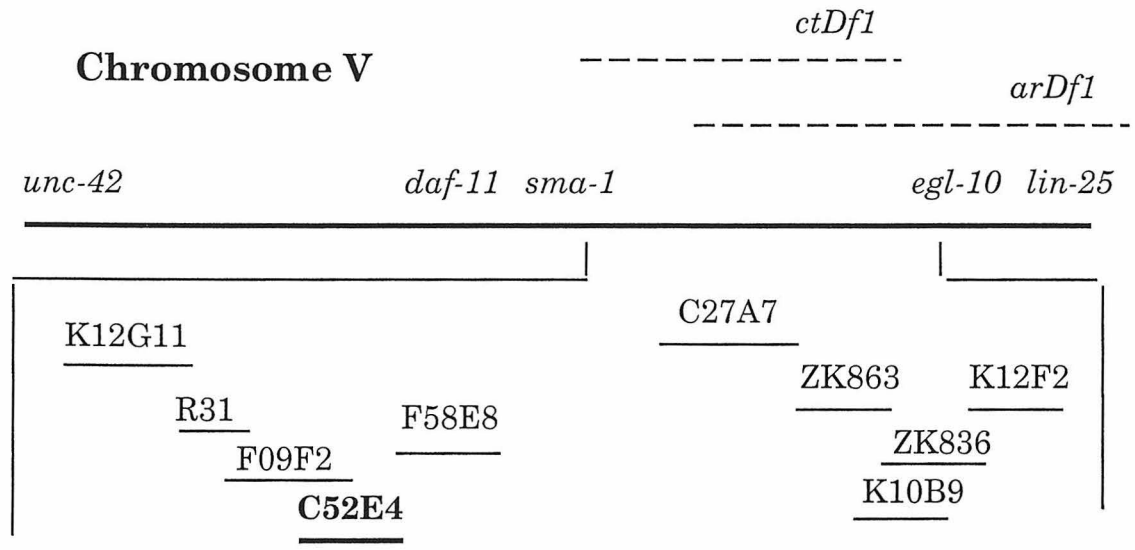
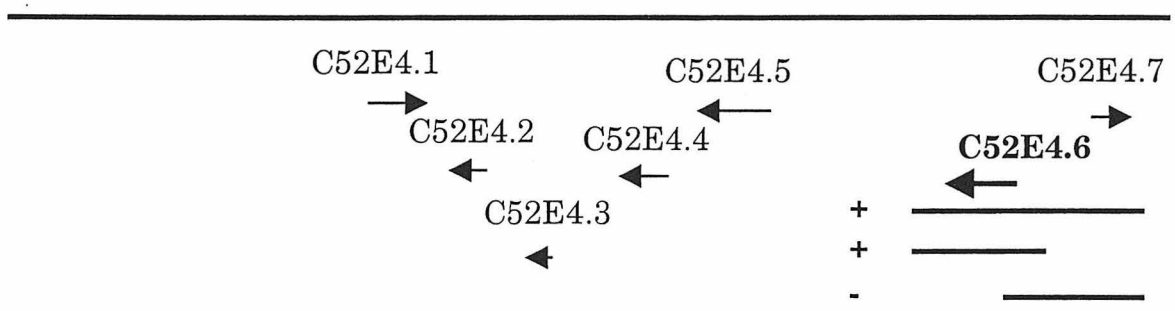


Figure 4

A. Genetic and physical map of *sag-4*



B. Subclone cosmid C52E4



C. Splicing pattern of *sag-4*

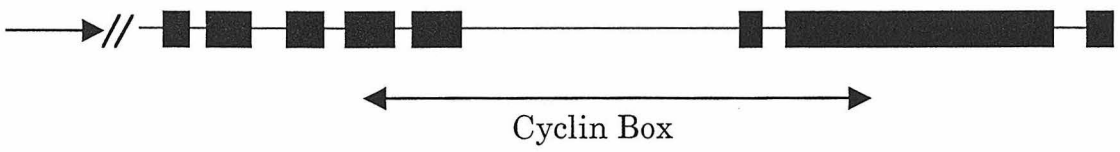
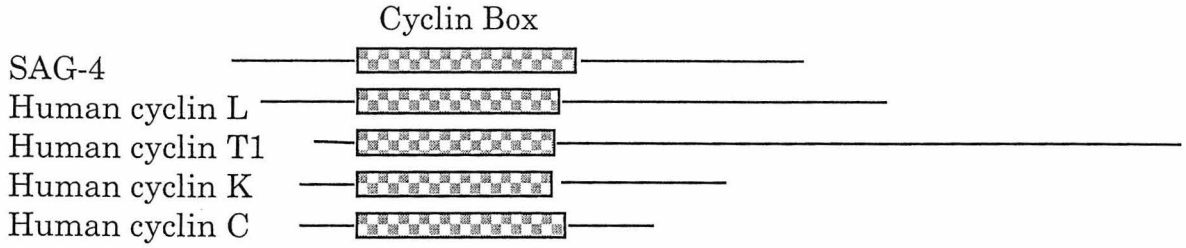


Figure 5

A. Cyclin Box in SAG-4 and homologous cyclins



B. Alignment of SAG-4 and homologous cyclins in the Cyclin Box

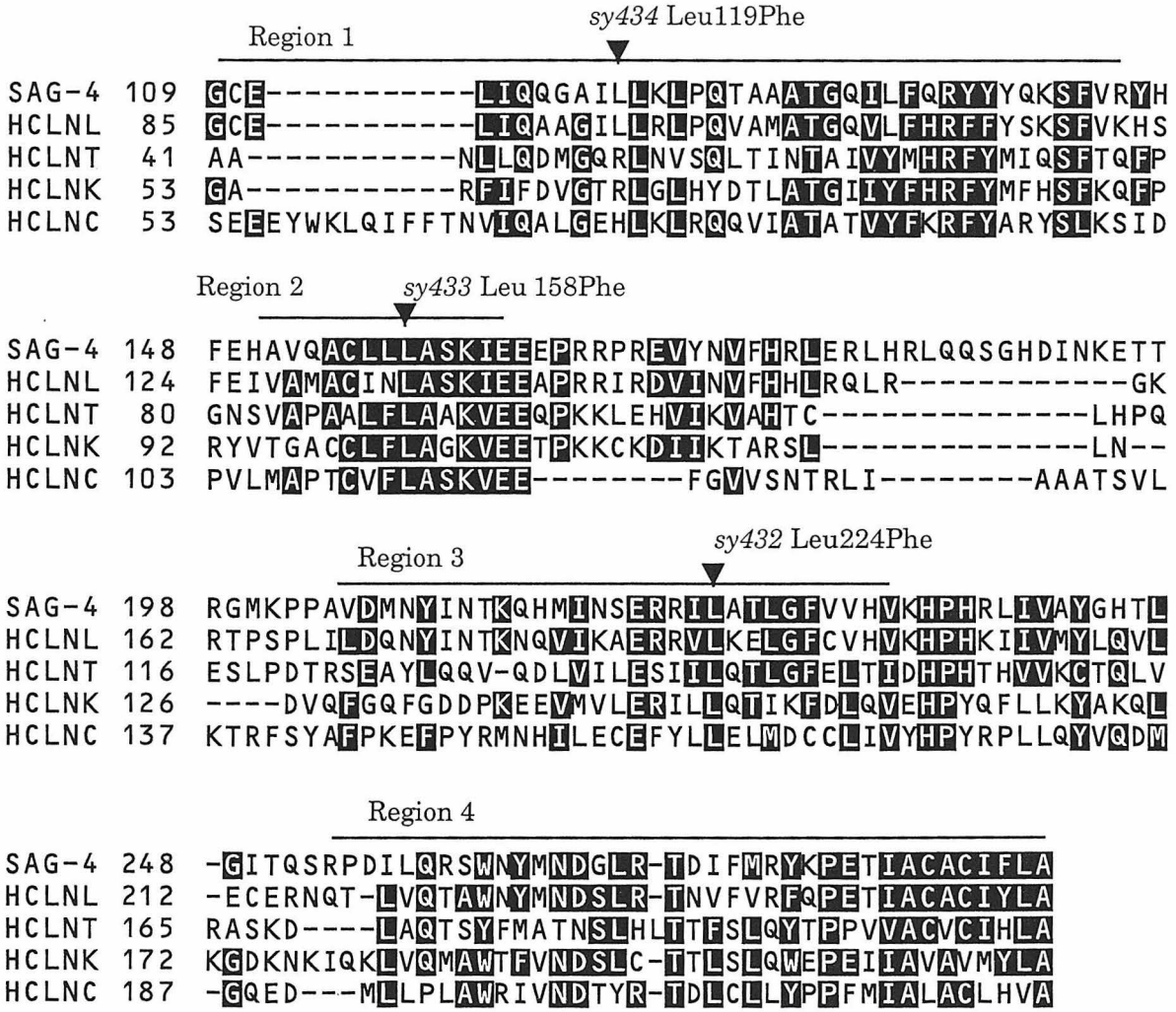


Figure 6

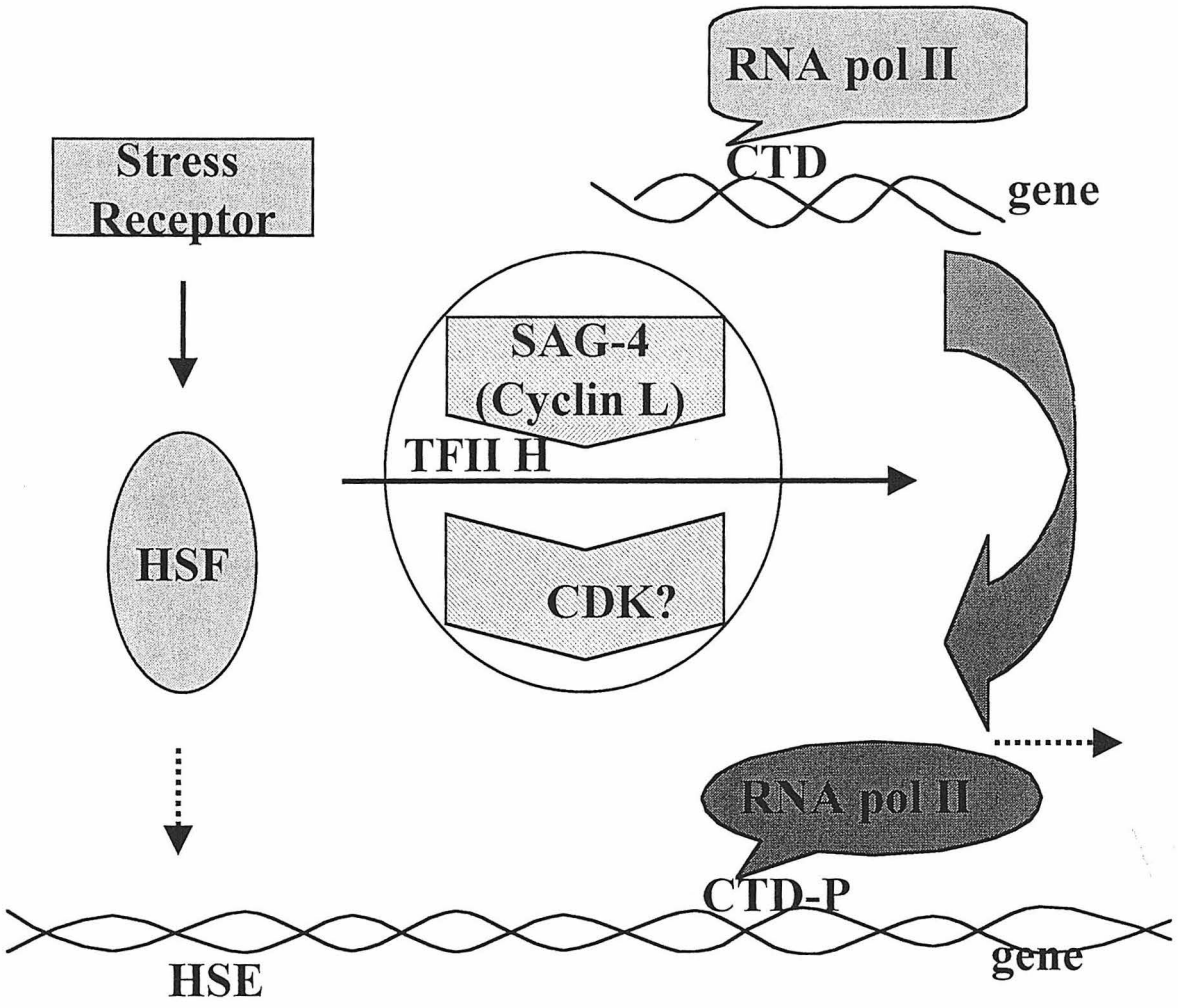

RNA pol II
CTD
gene
Stress
Receptor
SAG-4
(Cyclin L)
TFII H
HSF
CDK?
RNA pol II
CTD-P
gene
HSE

Table 1A: suppressor screen for activated Goα under heat shock promoter

Classes	Genes	Alleles	Location	H.S. GOA-1	Phenotypes
I	*sag-1(a.k.a. dgk-1)*	14	X L	unaffected	hyperactive and Egl-c
	sag-2(p.k.a. eat-16)	1	I C	unaffected	hyperactive and Egl-c
II	*sag-4*	3	V C	partially decrease	wild type appearance
	sag-8	1	?	partially decrease	wild type appearance
III	*sag-3*	1	I R	completely decrease	Egl-d
	sag-5	2	III L	completely decrease	variable, Egl-d
IV	*sag-6*	1	V	?	wild type appearance
V	*sag-7*	1	X	?	lethargic, Egg-d, small brood size

Table 1B. Double mutants between Class II and Class I suppressors

Genotype	Before H.S.		After H.S.	
(All in *syIs17* background)	% animals laid >3eggs/hour	eggs/hour	% animals laid >3 eggs/hour	eggs/hour
Control	90%	3.7±1.1	0%	0
dgk-1(sy428)	90%	2.9±0.7	0%	0.05±0.11
sag-4(sy433)	100%	5.6±1.2	0%	0.23±0.31
sag-8(sy530)	60%	3.2±2.8	0%	0.63±0.90
dgk-1(sy428); sag-4(sy433)	100%	4.3±0.8	60%	2.5±0.40

Table 2: *sag-4* only suppress heat-shock promoter

strain	promoter	protein	suppression
syIs9	*goa-1*	Go(Q205L)	No
syIs2	*lin-3*	LIN-3LacZ	No
let-2	*let-2*	LET-2	No
syIs17	*hsp16-2*	Go(Q205L)	Yes
syIs38	*hsp16-2*	Gq(Q205L)	Yes
syIs46	*hsp16-2*	GFP	Yes

Table 3: Cyclin families from *C. elegans* , *Drosophila* and Human

(Shown is the identity between the whole protein sequences)

	C. elegans				*Drosophila*				human			
	cyclin L	**cyclin T**	**cyclin K**	**cyclin C**	**cyclin L**	**cyclin T**	**cyclin K**	**cyclin C**	**cyclin L**	**cyclin T1**	**cyclin K**	**cyclin C**
(*C. elegans*)												
cyclin L	100%	20%	26%	23%	**36%**	25%	25%	26%	**39%**	25%	24%	25%
cyclin T		100%	29%	21%	25%	**26%**	23%	21%	22%	**29%**	23%	25%
cyclin K			100%	20%	31%	29%	**43%**	28%	30%	30%	**39%**	31%
cyclin C				100%	19%	22%	25%	**40%**	24%	22%	28%	**38%**
(*Drosophila*)												
cyclin L					100%	26%	25%	27%	**55%**	24%	27%	28%
cyclin T						100%	29%	27%	24%	**35%**	30%	26%
cyclin K							100%	27%	28%	28%	**57%**	31%
cyclin C								100%	27%	25%	29%	**72%**
(Human)												
cyclin L									100%	25%	26%	29%
cyclin T1										100%	30%	24%
cyclin K											100%	29%
cyclin C												100%

Chapter 3:

Antagonism between $G_o\alpha$ and $G_q\alpha$ in *C. elegans*:

The RGS Protein EAT-16 is necessary for $G_o\alpha$ signaling and regulates $G_q\alpha$ Activity

Antagonism between $G_o\alpha$ and $G_q\alpha$ in *Caenorhabditis elegans*: the RGS protein EAT-16 is necessary for $G_o\alpha$ signaling and regulates $G_q\alpha$ activity

Yvonne M. Hajdu-Cronin,[1,3] Wen J. Chen,[1,3] Georgia Patikoglou,[2] Michael R. Koelle,[2] and Paul W. Sternberg[1,4]

[1]Howard Hughes Medical Institute (HHMI) and Division of Biology, California Institute of Technology, Pasadena, California 91125 USA; [2]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520 USA

To elucidate the cellular role of the heterotrimeric G protein G_o, we have taken a molecular genetic approach in *Caenorhabditis elegans*. We screened for suppressors of activated GOA-1 ($G_o\alpha$) that do not simply decrease its expression and found mutations in only two genes, *sag-1* and *eat-16*. Animals defective in either gene display a hyperactive phenotype similar to that of *goa-1* loss-of-function mutants. Double-mutant analysis indicates that both *sag-1* and *eat-16* act downstream of, or parallel to, $G_o\alpha$ and negatively regulate EGL-30 ($G_q\alpha$) signaling. *eat-16* encodes a regulator of G protein signaling (RGS) most similar to the mammalian RGS7 and RGS9 proteins and can inhibit endogenous mammalian G_q/G_{11} in COS-7 cells. Animals defective in both *sag-1* and *eat-16* are inviable, but reducing function in *egl-30* restores viability, indicating that the lethality of the *eat-16; sag-1* double mutant is due to excessive $G_q\alpha$ activity. Analysis of these mutations indicates that the G_o and G_q pathways function antagonistically in *C. elegans*, and that $G_o\alpha$ negatively regulates the G_q pathway, possibly via EAT-16 or SAG-1. We propose that a major cellular role of G_o is to antagonize signaling by G_q.

[*Key Words*: *C. elegans*; G_o protein; G_q protein;RGS protein; signaling; regulation]

Received April 26, 1999; revised version accepted June 2, 1999.

$G_o\alpha$, a member of the G_i subfamily, is the major heterotrimeric G protein α-subunit of the brain and exists only in species with a nervous system. Although $G_o\alpha$ homologs have been isolated biochemically from several species, including cow (Sternweis and Robishaw 1984; Van Meurs et al. 1987), *Drosophila* (Yoon et al. 1989), *Xenopus* (Olate et al. 1989), hamster (Hsu et al. 1990), and man (Lavu et al. 1988), little is known about the mechanisms through which $G_o\alpha$ functions. To elucidate these mechanisms, we are studying *Caenorhabditis elegans* $G_o\alpha$ (GOA-1), which is 81%–82% identical to mammalian homologs (Lochrie et al. 1991) and is expressed throughout the entire *C. elegans* nervous system (M.R. Koelle, unpubl.; Mendel et al. 1995; Ségalat et al. 1995) and apparently also in some muscles (Mendel et al. 1995; Ségalat et al. 1995). GOA-1 modulates many behaviors, including locomotion and egg laying: mutants defective in *goa-1* function display hyperactive egg-laying and locomotion behaviors, whereas transgenic animals overexpressing wild-type or constitutively activated GOA-1 are lethargic and egg-laying defective (Mendel et al. 1995; Ségalat et al. 1995). Heat shock-induced expression of activated GOA-1 results in lethargy at any developmental stage, indicating that GOA-1 can function throughout the life span of the worm (Mendel et al. 1995).

G protein subunits can function as switches in signal transduction (Simon et al. 1991; Hepler and Gilman 1992). When inactive, the Gα-subunit is bound to GDP and associated with the Gβγ-subunits. Upon activation of an associated transmembrane receptor by a ligand, the α-subunit exchanges GDP for GTP and dissociates from βγ. In this state, the α-subunit is free to interact with effector molecules. GTP hydrolysis inactivates the α-subunit, returning it to Gβγ. Free βγ can also interact with effectors (Birnbaumer 1992). Substitution of leucine for a glutamine in a residue required for GTPase activity (Q205L for GOA-1 and EGL-30) renders the Gα-subunit constitutively activated (Graziano and Gilman 1989).

GOA-1 activity is thought to be regulated by EGL-10 (Trent et al. 1983), which along with the yeast *SST2* gene and GAIP first defined the RGS family of proteins (de Vries et al. 1995; Dohlman et al. 1996; Koelle and Hor-

[3]These authors contributed equally to this work.
[4]Corresponding author.
E-MAIL pws@cco.caltech.edu; FAX (626) 568-8012.

vitz 1996). RGS proteins negatively regulate G protein activity (Arshavsky and Pugh 1998; Berman and Gilman 1998) by acting as GTPase activating proteins (GAPs) for Gα-subunits, stabilizing the transition state during hydrolysis, and facilitating a rapid return to the inactive state (Berman et al. 1996a; Hunt et al. 1996; Watson et al. 1996; Faurobert and Hurley 1997). *egl-10* loss-of-function mutant animals have the opposite phenotype as *goa-1* loss-of-function mutants. Eliminating EGL-10 function in a mutant lacking GOA-1 has no additional phenotypic effect, suggesting that EGL-10 may act as a GAP specific for $G_o\alpha$ in *C. elegans* (Koelle and Horvitz 1996). So far, 19 mammalian RGSs have been found (Berman and Gilman 1998), and the *C. elegans* genome project has identified 12 genes containing the RGS core domain (Sulston et al. 1992; the *C. elegans* Sequencing Consortium 1998). Whereas EGL-10 may be an RGS for *C. elegans* $G_o\alpha$, an RGS that regulates *C. elegans* $G_q\alpha$ has not yet been identified.

To identify components in $G_o\alpha$-mediated signaling, we performed random mutagenesis looking for suppressors of constitutively activated $G_o\alpha$ in *C. elegans*, and we isolated mutations in two loci that appear to act downstream of, or parallel to, $G_o\alpha$ based on epistasis analysis: *sag-1*, a new locus, and *eat-16* (Avery 1993). Here, we present an analysis of the function of *eat-16*. We positionally cloned *eat-16* and found that it encodes an RGS homolog with an expression pattern similar to that of GOA-1. Based on double- and triple-mutant analysis involving $G_o\alpha$, $G_q\alpha$, *sag-1*, and *eat-16*, we believe that EAT-16 functions as an RGS for $G_q\alpha$ and that $G_o\alpha$ may negatively regulate $G_q\alpha$-mediated signaling in egg laying and locomotion. Consistent with our in vivo genetic data, EAT-16 can down-regulate the endogenous mammalian G_q/G_{11} when transfected into COS-7 cells. SAG-1 strongly inhibits $G_q\alpha$-mediated signaling and may function downstream of, or parallel to, $G_q\alpha$.

Results

Isolation of sag-1 *and* eat-16 *mutations as suppressors of activated GOA-1*

We performed a genetic screen for extragenic suppressors of *syIs17*, an integrated transgene expressing the constitutively activated *goa-1[Q205L]* mutant gene under the control of a heat shock promoter (hs-G_oQL). Upon heat shock, *syIs17* animals progressively cease locomotion, foraging, feeding, and production and laying of eggs (Mendel et al. 1995). Animals were mutagenized with either ethylmethanesulfonate (EMS; 21,000 haploid genomes) or trimethylpsoralen and UV irradiation (11,000 haploid genomes). The grandprogeny of mutagenized animals were heat-shocked as adults, and moving or foraging mutants were selected. In this manner, 15 independent suppressor strains were isolated that displayed a hyperactive phenotype (see below) in addition to suppressing *syIs17[hs-G_oQL]* (Fig. 1). Fourteen of these mutations mapped to the same region and failed to complement one another, defining a new locus, *sag-1* (suppressor of activated G protein). The other mutation, *sy438*, was allelic to *eat-16(ad702)*, which was isolated previously in a screen for defects in pharyngeal pumping (Avery 1993). *ad702*, the original mutation defining *eat-16*, could also suppress the heat shock-induced lethargy of *syIs17[hs-G_oQL]*, as could *sy438/ad702 trans*-heterozygotes (data not shown).

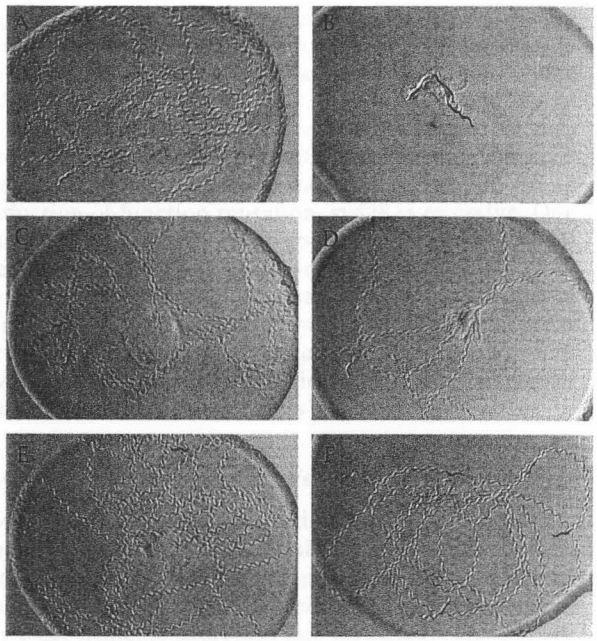


Figure 1. *sag-1* and *eat-16* mutations suppress the lethargy caused by heat shock of *syIs17[hsp::goa-1(QL)]*. Five adult worms were placed in the center of a bacterial lawn, allowed to crawl for 5 min, and photographed. Animals were then heat shocked and photographed 3 hr later. (*A*) *dpy-20 syIs17* animals without heat shock. (*B*) *dpy-20 syIs17* animals after heat shock treatment. (*C*) *dpy-20 syIs17; sag-1(sy428)*. (*D*) The same *dpy-20 syIs17; sag-1(sy428)* animals after heat-shock treatment. (*E*) *eat-16(sy438); dpy-20 syIs17*. (*F*) The same *eat-16(sy438); dpy-20 syIs17* animals after heat shock treatment.

Linkage tests eliminated the possibility that the suppression of hs-G_oQL could have been due to a deletion of the *syIs17* locus. All 14 *sag-1* mutations were X-linked and resided close to *unc-1*. Three-factor mapping placed *sag-1(sy428)* between *unc-1* and *egl-17*, whereas *eat-16(sy438)* mapped to linkage group (LG) I between *unc-29* and *lin-11* (see Fig. 2; Materials and Methods). In contrast, *syIs17* maps to LG IV (J. Mendel, pers. comm.).

sag-1 *and* eat-16 *mutants resemble* goa-1(lf) *mutants*

sag-1 and *eat-16* mutations not only suppressed the lethargy of hs-G_oQL (Fig. 1) but in a wild-type background conferred a phenotype similar to that of *goa-1* loss-of-function mutants (Mendel et al. 1995; Ségalat et al. 1995). Mutants laid eggs hyperactively, that is, soon after fertilization, resulting in eggs laid as early as uncleaved (Table 1). In addition, eggs were produced more slowly than the wild type (data not shown), resulting in uteri devoid of eggs (Table 1). Forward locomotion of *sag-1* and

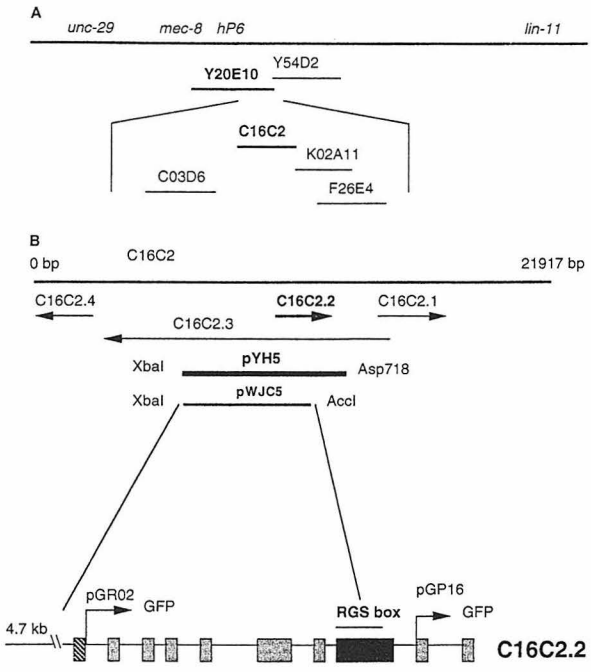


Figure 2. Positional cloning of *eat-16*. (*A*)Physical map of *eat-16*. *Eat-16* was mapped to the *left* half of the interval between *unc-29* and *lin-11* and to the *right* of *hP6* (see Materials and Methods). Two YACs covering this region were injected into *syIs17 dpy-20; eat-16(sy438); lin-15(n765)*. The *eat-16* phenotype was rescued by Y20E10 but not Y54D2. Four cosmids between *hP6* and the *right* end of Y20E10 were tested, and C16C2 rescued *eat-16(sy438)*. (*B*)Subclones of cosmid C16C2; cDNA map of *eat-16* and GFP constructs. Cosmid C16C2 has four open reading frames (*C. elegans* Sequencing Consortium 1998). The rescuing plasmid pYH5 contains the full sequence of C16C2.2 cloned into pBluescript; the first two exons of C16C2.3 are not included. The plasmid pWJC5 is an *XbaI–AccI* fragment from pYH5 cloned into pBluescript; it includes the same promoter region as pYH5, but the coding sequence of EAT-16 is terminated in the middle of RGS domain at amino acid 352. Full-length cDNA sequence of *eat-16* was obtained from clone yk356b3. *eat-16* has 10 exons; the RGS domain is contained within Exon 8, the largest exon. Reporter construct pGP16 includes 4.7 kb of the upstream promoter region and contains most of the *eat-16* coding region, including the RGS domain. Reporter construct pGR02 contains the upstream promoter region but only the first coding exon of *eat-16*.

eat-16 mutants was more rapid than wild type (Table 1). Conversely, pharyngeal pumping rates were impaired (Table 1); therefore, *sag-1* and *eat-16* mutants were somewhat starved and had a pale, scrawny appearance. Thus, the phenotypes of *sag-1* and *eat-16* mutants indicated that these genes might function in a G_o-mediated signaling pathway.

goa-1(n363) null mutants crawl backwards with deeply exaggerated flexions compared with their forward locomotion (J. Mendel, unpubl.); this behavior was not observed in other *goa-1* mutants (J. Mendel, unpubl.) or in *sag-1(sy428)* or *eat-16* mutants. Either the *n363* deletion, which removes more than the entire *goa-1* coding region (Ségalat et al. 1995), also removes a neighboring gene responsible for this phenotype, or the behavior is mediated via a different mechanism than that involving SAG-1 or EAT-16.

Of the 14 *sag-1* mutations, *sy428* was used as the reference allele for all experiments: it displays a strong hyperactive phenotype and appears to be a null or strong reduction-of-function mutation. *sy428* is recessive: One hundred percent of heterozygotes were wild type in appearance and had at least seven eggs in their uteri ($n = 90$), and *sy428/Df* heterozygous animals display a similar phenotype to that of *sy428* homozygotes (see Materials and Methods). *eat-16(ad702)* and *eat-16(sy438)* have similar phenotypes (Table 1) and are reduction-of-function mutations (see below).

SAG-1 and EAT-16 do not affect GOA-1 expression

syIs17[hs-G_oQL] was selected as the parent strain for mutagenesis because of its stability and ease of culture; however, mutations might suppress hs-G_oQL by affecting heat shock-induced protein expression in general. Two experiments addressed this possibility. First, Western blot analysis indicated that mutations in *sag-1* or *eat-16* do not lower heat shock-induced GOA-1 expression (data not shown). Second, we examined the ability of *sag-1(sy428)* and *eat-16(sy438)* mutations to suppress activated GOA-1 under control of its normal regulatory sequences rather than a heat shock promoter. Both *sag-1(sy428)* and *eat-16(sy438)* suppressed the lethargy of *syIs9*, an integrated transgene of $G_o\alpha$ [Q205L] under control of the *goa-1* promoter (G_oQL; Mendel et al. 1995; Table 1). These experiments suggested that SAG-1 and EAT-16 function in GOA-1 signaling rather than in GOA-1 expression.

SAG-1 and EAT-16 function downstream of, or parallel to, GOA-1

eat-16(sy438) suppressed the lethargy and egg-laying defect of *syIs9[G_oQL]* (see above; Table 1), indicating that EAT-16 functions downstream of, or parallel to, GOA-1 and is required for GOA-1 signaling in both behaviors. Suppression of the G_oQL locomotory defect by *sag-1(sy428)* was similarly robust (Table 1), indicating that SAG-1 likely functions downstream of GOA-1 at least with respect to locomotion. *syIs9[G_oQL]; sag-1(sy428)* also laid fewer late-stage eggs than did *syIs9[G_oQL]*, but the egg-laying defect was only partially suppressed (Table 1).

In addition to suppressing activated $G_o\alpha$, *sag-1(sy428)* also significantly suppressed the egg-laying and locomotory defects of reduction-of-function mutations in *egl-30*, a *C. elegans* $G_q\alpha$ homolog that acts antagonistically to $G_o\alpha$, either in parallel or downstream (see below). In all cases, suppression of *egl-30* by *sag-1(sy428)* was stronger than that by *eat-16(sy438)* (Table 2; see below). We infer that SAG-1 and EGL-30 act on a common process and that SAG-1 functions downstream of, or parallel to, EGL-30.

Table 1. *Genetic characterization of* sag-1 *and* eat-16 *mutants*

Strain	Egg-laying phenotype: cells per egg	eggs (no.)	eggs in uterus	animals (no.)	Forward locomotion (sine waves/min)	Animals (no.)	Feeding (pumps/min)	Animals (no.)
Wild type (N2)	>10	50	12 ± 1.8	10	25.3 ± 4.9	10	218 ± 21	10
sag-1(sy428)	3.0 ± 1.4	50	1.5 ± 0.9	10	37.4 ± 8.7	10	161 ± 43	10
eat-16(sy438)	2.2 ± 1.1	50	1.3 ± 0.7	10	41.8 ± 6.8	12	156 ± 37	10
sy438/ +	>10	32	13 ± 3	6	N.D.		N.D.	
eat-16(ad702)	2.2 ± 1.0	50	2.8 ± 2.0	10	51.3 ± 8.9	11	110 ± 30	10
ad702/ +	>10	30	12 ± 3	8	N.D.		N.D.	
syIs9 [GoQ205L]	100% late[a]	25	16 ± 4.4	30	12.1 ± 5.1	9	N.D.	
eat-16(sy438); syIs9	0% late[a]	10	7.4 ± 2.9	5	35.9 ± 11.5	10	N.D.	
syIs9; sag-1(sy428)	85% late[a]	27	6.6 ± 2.2	30	40.0 ± 8.1	9		

N.D.Animals were assayed as described in Materials and Methods.
(N.D.) Not determined.
[a]Late-stage eggs are defined as having at least 50 cells (see Materials and Methods).

eat-16 encodes an RGS homolog

To understand the function of *eat-16* in $G_o\alpha$-mediated signaling, we positionally cloned it by transformation rescue. We mapped *eat-16* to the left half of the *unc-29 lin-11* interval and to the right of *mec-8* and *hP6* (Fig. 2A; see Materials and Methods). Rescue was obtained with Y20E10, one of two YACs tested that reside in the left part of the interval between *hP6* (Starr et al. 1989; Lundquist and Herman 1994) and *lin-11* (Freyd et al. 1990), and with C16C2, one of four cosmids tested. C16C2 contains four predicted open reading frames, including C16C2.2, an RGS homolog. C16C2.2 resides entirely within one large intron of its oppositely oriented neighbor C16C2.3 (Fig. 2B). Injection of pYH5, a 7-kb *Asp*718–*Xba*I subclone containing the promoter region and all of C16C2.2 (Fig. 2B) rescued the *eat-16(sy438)* mutant phenotype.

All members of the RGS family have a designated 120-amino-acid RGS core domain (Tesmer et al. 1997). Some of them (RGS7, RGS9, EGL-10, and RGS11) are also highly conserved throughout the amino terminal region, which includes the DEP domain and the GGL domain. The function of the DEP domain is unknown, but the GGL domain is ~34% identical to Gγ and can bind with Gβ in vitro (Snow et al. 1998). Full-length cDNA sequence of C16C2.2 was obtained from the clone yk356b3, a gift from Yuji Kohara (National Institute of Genetics, Mishima Japan. Sequence analysis indicates that C16C2.2 contains all three domains, making it most similar to RGS7, RGS9, and EGL-10 (Fig. 3).

To determine whether the RGS domain of C16C2.2 is necessary to rescue the *eat-16* mutant phenotype, we constructed pWJC5, a truncated genomic clone of *eat-16* that lacks amino acids 353–474. The carboxy-terminal part of the RGS domain (where *sa609* and *sy438* are located; see below) is deleted in this construct (Fig. 2B). Injection of this plasmid failed to rescue the *eat-16* phenotype, indicating that the RGS domain of C16C2.2 is required to suppress the phenotype of activated $G_o\alpha$, and the loss of it causes hyperactive egg laying and locomotion.

sy438 *and* ad702 *are reduction-of-function alleles of eat-16*

To verify that *eat-16* encodes the C16C2.2 RGS protein, we amplified and sequenced C16C2.2 genomic DNA from each *eat-16* mutant (see Materials and Methods). Each strain contained a single point mutation that was then confirmed by sequencing the opposite strand of DNA in the region of the mutation (Fig. 3). *sy438* is a missense mutation that changes a conserved serine at position 400 in the RGS domain to a phenylalanine. *ad702* is an AG → AA mutation in the splice acceptor site before the fourth exon, which is predicted to result in early termination before the RGS domain, although some properly spliced message is likely produced (see Aroian et al. 1993). We also sequenced two other alleles of *eat-16*, *sa609* and *sa735*, kindly provided by M. Robatzek and J. Thomas (University of Washington, Seattle). *sa735* is an AG → AA mutation in the splice acceptor site before the eighth exon (which contains the RGS domain), and *sa609* is another missense mutation within the RGS domain that changes a conserved arginine at position 396 to a cysteine. Both *sa609* and *sa735* confer a phenotype similar to *sy438* and *ad702* (data not shown).

Because the missense mutations confer a phenotype similar to the splice acceptor site mutations, they likely reduce EAT-16 function. Although *sy438* is essentially recessive (Table 1), we noticed that 6.5% of *sy438/+* heterozygotes looked like *sy438* homozygotes (n = 92). To test whether this effect was due to semidominance or haploinsufficiency, we examined by Nomarski optics animals heterozygous for the deficiency chromosome *ces-1(n703d) qDf9* (Ellis and Kimble 1995), which deletes *eat-16* and found that 58% of these animals had fewer than six eggs in their uteri and these eggs contained eight or fewer cells (n = 60 animals), indicating that the animals were laying eggs hyperactively. When placed in *trans* to *qDf9*, both *sy438* and *ad702* were viable and similar in phenotype to *sy438* and *ad702* homozygotes (data not shown). In contrast, animals bearing

Table 2. *Suppression of* egl-30 *mutant phenotypes by* sag-1 *and* eat-16

Strain	Egg-laying defects						Forward locomotion (sine waves/min)	Animals (no.)
	late-stage eggs (%)[a]	eggs (no.)	eggs about to hatch (%)	eggs (no.)	eggs retained in uterus	animals (no.)		
egl-30(ad805)	100	6[b]	83	6[b]	26.1 ± 5.8	18	N.A.[c]	
ad805 eat-16	75	8[b]	0	8[b]	27.1 ± 6.6	14	N.A.[c]	
ad805; sag-1	4	25	0	25	17.6 ± 4.0	19	17.8 ± 5.1	5
egl-30(ad809)	63	35	0	35	24.2 ± 2.9	21	N.A.[c]	
ad809 eat-16	37	38	0	38	21.6 ± 3.7	22	N.A.[c]	
ad809; sag-1	0	40	0	40	7.2 ± 1.6	21	26.2 ± 5.8	12
egl-30(md186)	77	13	31	13	22.9 ± 4.8	21	10.1 ± 3.7	9
md186 eat-16	20	35	0	35	22.0 ± 3.6	20	14.0 ± 4.7	11
md186; sag-1	3	35	0	35	13.9 ± 3.4	20	19.8 ± 4.1	6
md186 eat-16; sag-1	0	50	0	50	9.4 ± 2.4	20	32.3 ± 8.7	10
egl-30(n686)	90	30	67	30	20.2 ± 4.9	20	16.7 ± 4.4	18
n686 eat-16	53	49	12	49	15.2 ± 3.3	20	29.4 ± 9.7	9
n686; sag-1	4	24	0	24	6.8 ± 2.0	18	35.4 ± 6.7	10

Strains were assayed as described in Materials and Methods. Double mutants were constructed with *sag-1(sy428)* and *eat-16(sy438)*.
[a]Late stage was defined as after the comma stage (see Materials and Methods).
[b]Eggs were laid infrequently; therefore they were difficult to harvest.
[c](N.A.) Not available; mutants made few or no sinusoidal waves.

multiple copies of wild-type EAT-16 in the transgene *syEx256* are somewhat egg-laying defective (data not shown). That presumed overexpression has a phenotype opposite that of *sy438* and *ad702* supports the conclusion that *sy438* and *ad702* reduce EAT-16 function.

The expression pattern of EAT-16 is similar to that of GOA-1

To examine expression, we made GFP translational fusions linking GFP either to the amino terminus or carboxyl terminus of *eat-16*. Reporter construct pGP16 (Fig. 2) contains a 7.4-kb *ApaI–Bam*HI genomic fragment (including the *eat-16* promoter region) and fuses to GFP-coding sequences in the ninth coding exon predicted by the cDNA sequence; this construct contains the entire amino-terminal region and the RGS domain. Examination of transgenic animals carrying pGP16 using confocal microscopy showed that EAT-16 is expressed in most or all neurons, including the hermaphrodite specific neuron (HSN) required for egg laying, as well as in the vulval and pharyngeal muscles (Fig. 4). Expression was also occasionally seen in the spermatheca and body wall muscles (data not shown). Similar expression patterns were seen when the GFP-coding sequences were fused to the first coding exon of *eat-16*. The expression pattern is consistent with the mutant phenotypes observed and is similar to the GOA-1 expression pattern (Mendel et al. 1995; Ségalat et al. 1995); therefore, *eat-16* might act in many of the same cells as $G_o\alpha$.

EAT-16 does not regulate GOA-1

RGS proteins have been shown to facilitate the inactivation of Gα-subunits by GTP hydrolysis (Berman et al. 1996a; Hunt et al. 1996; Watson et al. 1996; Faurobert and Hurley 1997); therefore, EAT-16 likely regulates one or more Gα-subunits in *C. elegans*. We thought it unlikely that $G_o\alpha$ would be the target for EAT-16 based on the following arguments. First, the *syIs17* parent strain expresses multiple copies of constitutively activated GOA-1(Q205L) upon heat shock, which would likely be insensitive to a wild-type RGS for G_o (Berman et al. 1996b). Reducing the function of this RGS would render the excess activated subunits even more immune to down-regulation and therefore would not suppress the hs-G_oQL phenotype. Second, if EAT-16 negatively regulates GOA-1, we would expect the *eat-16* reduction-of-function phenotype to resemble that of activated $G_o\alpha$; instead, *eat-16* mutants resemble *goa-1* hypomorphs. Finally, *eat-16* appears to act downstream of, or parallel to, *goa-1* based on double-mutant analysis with *syIs9[G_oQL]*.

However, the similar expression patterns of EAT-16 and GOA-1 encouraged us to further test the possibility that EAT-16 regulates GOA-1. We overexpressed wild-type EAT-16 in a *goa-1* null mutant background and found that the transgene *syEx256[eat-16(+)]* suppressed the hyperactive egg-laying behavior of *goa-1(n363)*: Ten percent of eggs laid by *goa-1; syEx256* animals were premature (n = 48), compared with 97% of eggs laid by their nontransgenic siblings (n = 34) (see Materials and Methods). If EAT-16 preferentially regulates GOA-1, we would have seen no suppression of *goa-1(n363)*, because the *n363* lesion deletes the entire *goa-1* coding region (Ségalat et al. 1995). Therefore, we conclude that the major function of EAT-16 is not to regulate GOA-1 activity.

Genetic evidence that EAT-16 regulates EGL-30 $G_q\alpha$

Because GOA-1 was an unlikely target for EAT-16, we considered other *C. elegans* Gα-subunits. Two of many

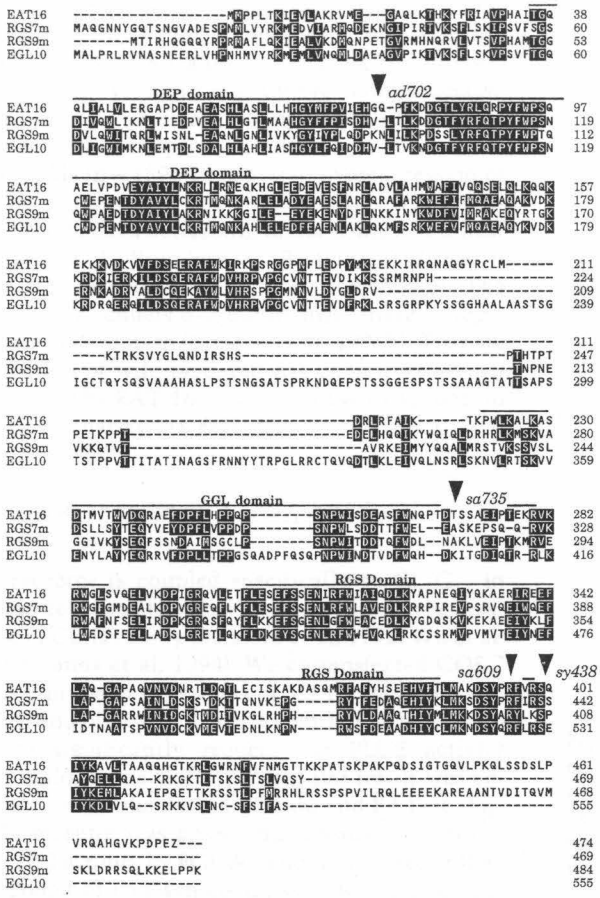


Figure 3. Sequence alignment of EAT-16 with RGS7, RGS9, and EGL-10. Regions of sequence similarity are highlighted. EAT-16 is 42% identical to mouse RGS7 in the RGS domain, 39% identical in the DEP domain, and 45% identical in the GGL domain; 41% identical to mouse RGS9 in the RGS domain, 37% identical in the DEP domain, and 34% identical in the GGL domain; and 30% identical to *C. elegans* EGL-10 in the RGS domain, 40% identical in the DEP domain, and 39% identical in the GGL domain. Arrowheads indicate the sites of *eat-16* mutations. *ad702* is AG → AA in the splice acceptor site before exon 4, *sa735* is AG → AA in the splice acceptor site before exon 8, *sa609* is Arg-396–Cys, and *sy438* is Ser-400–Phe.

Gα-subunits identified in *C. elegans* have been shown to affect the same sets of behaviors as $G_o\alpha$: EGL-30, the $G_q\alpha$ homolog (Brundage et al. 1996), and GSA-1, the $G_s\alpha$ homolog (Korswagen et al. 1997). Because the crystal structure of $G_s\alpha$ as well as biochemical evidence suggests that $G_s\alpha$ is not regulated by an RGS (Berman et al. 1996b; Tesmer et al. 1997; Natochin and Artemyev 1998), we focused our attention on $G_q\alpha$. Whereas the putative null mutation, *ad810*, is lethal (Brundage et al. 1996), reduction-of-function mutations in *egl-30* result in a lethargic and egg laying-defective phenotype roughly opposite to that of *goa-1* reduction-of-function or null mutations. *ad809* is a splice-donor site mutation, and *ad805* and *n686* are splice-acceptor site mutations; all result in reduced copies of full-length EGL-30 (Brundage et al. 1996). *md186* is a missense mutation that reduces EGL-30 activity (Miller et al. 1996; L. Brundage, pers. comm.). The phenotypes of these reduction-of-function mutants vary in severity with *ad805* having the strongest phenotype (Brundage et al. 1996). Because *egl-30* and *eat-16* reduction-of-function mutations have essentially opposite phenotypes, we asked whether EAT-16 might regulate EGL-30 activity.

We reasoned that if EAT-16 accelerates $G_q\alpha$ GTPase activity, reducing EAT-16 function should allow more $G_q\alpha$-subunits to remain active, thereby alleviating the phenotype of a $G_q\alpha$ hypomorph, whereas reducing EAT-16 function in a null $G_q\alpha$ background should have no phenotypic effect. To test this hypothesis, we built double mutants between *eat-16(sy438)* and several *egl-30* mutations. Although *sy438* did not suppress the lethality of the putative null allele *ad810* (see Materials and Methods), we found that *sy438* partially suppressed the egg-laying defect of all hypomorphs tested and partially suppressed the locomotory defect of *n686* (Table 2). These results support the hypothesis that EAT-16 inhibits EGL-30 activity.

To examine whether multiple copies of EAT-16 could compensate for EGL-30 overexpression, we overexpressed EAT-16 (using the transgene *syEx256*) in two different *egl-30* transgenic strains (see Materials and Methods). Animals bearing *syIs36[egl-30(+)]*, an integrated transgene overexpressing wild-type EGL-30 (L. Brundage, pers. comm.), move and lay eggs hyperactively (see

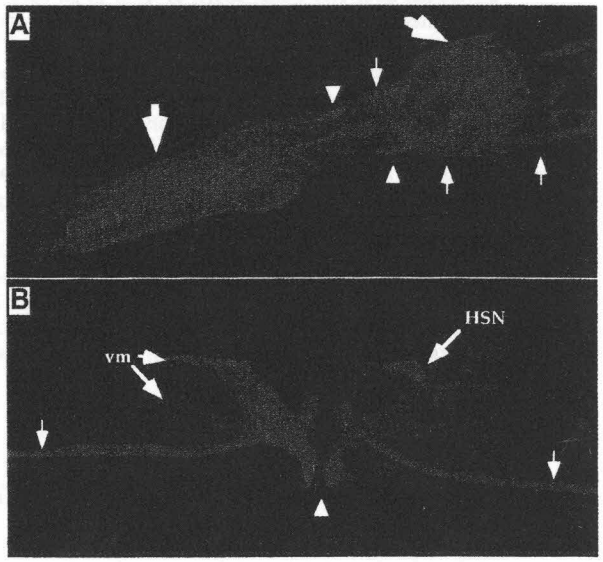


Figure 4. Expression of *eat-16* in *C. elegans*. Transgenic animals carrying the *eat-16*::GFP reporter construct pGP16 were examined by confocal fluorescence microscopy. Similar expression patterns were seen using the reporter construct pGR02. (*A*) Adult head region, showing fluorescence in the cell bodies (arrowheads) and processes (small arrows) of many neurons, as well as in pharyngeal muscles (large arrows). (*B*) Adult vulva region. Vulval opening is indicated by the arrowhead. Fluorescence is detected in the HSN neuron, vulva muscles (vm), and ventral cord neurons (small arrows).

Brundage et al. 1996). *syIs36/+; syEx256* transgenic animals displayed various phenotypes (probably due to mosaicism of the *syEx256* transgene), ranging from hyperactive (similar to *syIs36*) to slightly egg-laying defective (similar to *syEx256*); however, suppression of the pale, scrawny phenotype of *syIs36[egl-30(+)]* was observed in 50% of animals (n = 189). In contrast, overexpression of EAT-16 did not suppress the phenotype of overexpression of activated EGL-30(Q205L) under control of a heat shock promoter (L. Brundage, C. Bastiani, P.W. Sternberg, and M.I. Simon, unpubl.; data not shown). The Q205L mutation renders α-subunits insensitive to regulation by an RGS protein (Berman et al. 1996b). That we see suppression of wild-type, but not constitutively activated, EGL-30 by EAT-16 is consistent with a model in which EAT-16 inactivates EGL-30.

EAT-16 reduces endogenous G_q/G_{11} activity in COS-7 cells

The M1 receptor is coupled specifically to G_q/G_{11} in mammalian cells, and the activity of G_q/G_{11} can be measured by a PLCβ-IP_3 assay (Berstein et al. 1992; Wu et al. 1992; Offermanns et al. 1994). We cotransfected COS-7 cells with expression constructs of M1 receptor and *eat-16* (Fig. 5A) and observed that the addition of EAT-16 to the system significantly reduces the PLCβ activity caused by endogenous G_q/G_{11}. A similar result was obtained when we cotransfected EGL-30 and EAT-16 (Fig. 5B; no M1 receptor was added), but because the stimulation of PLCβ activity by EGL-30 is not much greater than the background of endogenous G_q/G_{11}, we cannot infer from this experiment that EAT-16 down-regulates EGL-30.

Reducing EGL-30 function restores viability to eat-16; sag-1 *mutants*

We constructed a strain that segregates *eat-16; sag-1* double mutants and found that >99% of the double mutants die (see Materials and Methods), arresting during larval development (Fig. 6A). Because each suppressor mutation results in a starved phenotype, one might argue that the lethality is caused by an additive starvation effect. However, *goa-1(n363)* has a more severe phenotype than either suppressor, and *goa-1(n363); sag-1(sy428)* double mutants are viable. Therefore SAG-1 and EAT-16 appear to act synergistically and are functionally redundant for survival.

Because *sag-1(sy428)* significantly suppresses the phenotype of *egl-30* hypomorphs (see above; Table 2), it seemed likely that SAG-1 and EAT-16 function synergistically to reduce EGL-30 signaling. If so, lowering EGL-30 signaling might suppress the lethality of *eat-16; sag-1* double mutants. To test this hypothesis we constructed the *egl-30(md186) eat-16(sy438); sag-1(sy428)* triple mutant (see Materials and Methods) and found that it is viable to adulthood (Fig. 6C); this result indicates that excess EGL-30 activity is responsible for the lethality of *eat-16; sag-1* double mutants. The lethargic and egg laying-defective phenotype of *egl-30(md186)* (Fig. 6B) was almost completely suppressed in the triple mutant (Table 2; Fig. 6C).

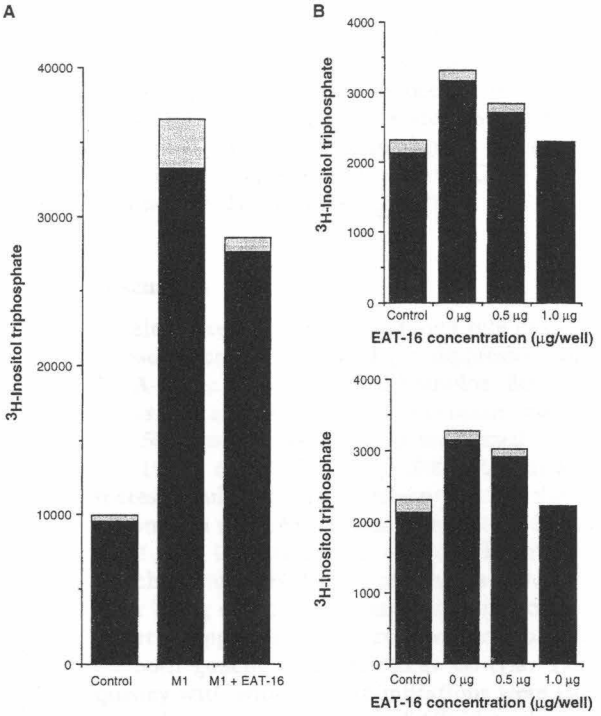


Figure 5. EAT-16 down-regulates the PLCβ activity of endogenous G_q/G_{11} in COS-7 cells. (*A*) M1 receptor cotransfection. COS-7 cells were transfected with either control vector pCIS (*left* lane), 0.25 µg of M1 receptor (middle lane), or 0.25 µg of M1 receptor + 0.25 µg of EAT-16 (*right* lane). The total concentration of DNA was normalized to 1.0 µg per well using pCIS vector. (The M1 receptor activates endogenous G_q/G_{11}.) Shown is the measured [^{3}H]Inositol phosphate level 48 hr after transfection. Higher concentrations of EAT-16 (up to 1.5 µg per well) gave similar results (data not shown). (*B*) EGL-30 cotransfection. EAT-16 was cotransfected at various concentrations with EGL-30 into COS-7 cells. Total DNA concentration was normalized to 2.0 µg per well with pCIS. (No M1 receptor was added in this experiment.) PLCβ activity was caused by both endogenous G_q/G_{11} and EGL-30. (*Top*) COS-7 cells were transfected with control vector pCIS (*left*), 0.5 µg of EGL-30 (second from *left*), or 0.5 µg of EGL-30 with various concentrations of EAT-16 (*right* lanes). (*Bottom*) Same conditions as at *top* except that 1.0 µg of EGL-30 was transfected. (Shaded bars) S.D.; (solid bars) average.

$G_o\alpha$ antagonizes $G_q\alpha$ in C. elegans

Reduction-of-function mutations in *goa-1* and *egl-30* have essentially opposite phenotypes (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996), suggesting that $G_o\alpha$ and $G_q\alpha$ function antagonistically in *C. elegans*. *egl-30(ad805) goa-1(n363)* double mutants are lethargic and egg laying-defective like *egl-30(ad805)* animals (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.), indicating that EGL-30 functions downstream of,

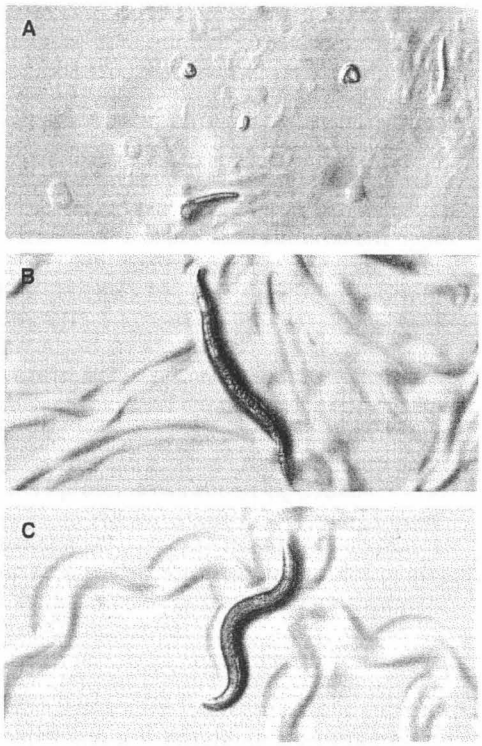


Figure 6. Reducing EGL-30 function restores viability to *eat-16; sag-1* double mutants. All animals were photographed at the same magnification. (*A*) *eat-16(sy438); sag-1(sy428)* double mutants arrest as young larvae. (*B*) *egl-30(md186)* mutants are le thargic and egg-laying defective and leave flattened tracks (Miller et al. 1996). (*C*) *egl-30(md186) eat-16(sy438); sag-1(sy428)* triple mutants are viable and active, leaving sinusoidal tracks and laying eggs.

or parallel to, GOA-1. Although on a gross level the double mutant resembled the *ad805* single mutant, *ad805 n363* had more active egg laying than *ad805* alone: Fifteen percent of *ad805 n363* eggs ($n = 34$) versus 100% of *ad805* eggs ($n = 12$) were laid >5 hr after fertilization, respectively. The partial suppression of *ad805* by *n363* might be due to the presumably low level of wild-type EGL-30 activity expressed by the splice acceptor site mutant *ad805* (especially because the *egl-30* null phenotype is lethal; Brundage et al. 1996), which would be enhanced when GOA-1 activity is reduced. Nonetheless, the partial suppression of the egg-laying defect of *egl-30(ad805)* by *goa-1(n363)* is similar to that by *eat-16(sy438)* (see Table 2), consistent with GOA-1 and EAT-16 both negatively regulating EGL-30.

As with reducing gene function, overexpression of wild-type or activated GOA-1 and EGL-30 have opposite phenotypic effects (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996). We reasoned that if the lethargy of *syIs17[hs-G_oQL]* is due to excessive negative regulation of $G_q\alpha$ activity by activated $G_o\alpha$, then simultaneously overexpressing $G_q\alpha$ might suppress this lethargy. We tested this hypothesis by overexpressing both Gα-subunits in wild-type animals (see Materials and Methods) and found that *syIs36[egl-30(+)]*, which overexpresses multiple copies of wild-type EGL-30, suppressed the heat shock-induced lethargy of *syIs17-[hsG_oQL]* (89 of 90 animals tested). Supporting these observations, our identification of genes involved in EGL-30 signaling as suppressors of activated GOA-1 suggests that $G_o\alpha$ negatively regulates $G_q\alpha$ activity in *C. elegans*.

Discussion

To elucidate the largely unknown role of $G_o\alpha$ in signal transduction, we screened for suppressors of activated GOA-1, the *C. elegans* $G_o\alpha$ homolog. Because in *C. elegans* loss-of-function mutations occur at a frequency of 1 in 5000 to 1 in 2000 EMS-mutagenized gametes (Brenner 1974), our screen of 21,000 EMS-mutagenized gametes should be fairly representative of the *C. elegans* genome. In this EMS screen we isolated 13 mutations in *sag-1* and 1 mutation in *eat-16*. The frequency with which we identified *sag-1* mutations is consistent with their being reduction-of-function mutations. Mutations of *eat-16* appear to be reduction-of-function alleles based on both genetic and molecular criteria. The low frequency with which *eat-16* mutations were isolated suggests that weak mutations in EAT-16 might not suppress *syIs17[hs-G_oQL]* well enough to be detected in our screen. In addition, Avery (1993) did not isolate any mutations in *eat-16* in an F_2 EMS mutagenesis of similar size; *ad702* was isolated in an F_1 screen designed to recover mutations at 100% efficiency (Avery 1993). These results and ours indicate that mutations in *eat-16* are not easily recoverable; perhaps only mutations affecting the RGS domain (one exon) confer starvation and suppression of hs-G_oQL. *eat-16* and *sag-1* mutants display a hyperactive phenotype similar to that of *goa-1* loss-of-function mutants and are required for GOA-1 signaling. The simplest interpretation of our results is that EAT-16 and/or SAG-1 function as effectors for GOA-1; however, it is also possible that the effectors of GOA-1 either do not mutate to a viable phenotype or are numerous and functionally redundant.

EAT-16 and EGL-10 distinguish between G_q and G_i/G_o subfamilies

Several lines of evidence indicate that EAT-16 does not inhibit $G_o\alpha$. *eat-16* reduction-of-function mutations suppress the phenotype of an overexpressed, constitutively activated form of GOA-1 and phenotypically resemble *goa-1* loss-of-function mutants; moreover, overexpression of EAT-16 compensates for a complete deletion of *goa-1*. We have presented genetic and biochemical data consistent with a model in which EAT-16 regulates the $G_q\alpha$ homolog EGL-30. A missense mutation in the RGS domain of *eat-16* alleviates the phenotypes of several *egl-30* reduction-of-function mutations but not that of the putative null allele *ad810*. Overexpression of EAT-16 can suppress the phenotype caused by overexpression of wild-type, but not constitutively activated, EGL-30. Co-

transfection of EAT-16 and M1 receptor in COS-7 cells significantly reduces PLCβ activity resulting from endogenous mammalian G_q/G_{11}, and a similar result was observed in cells cotransfected with EAT-16 and EGL-30. These results taken together argue that EAT-16 functions as a GAP for EGL-30.

EGL-10 and EAT-16, which are homologous to each other in both the amino-terminal region and the RGS domain, have similar GFP expression patterns but opposite phenotypic effects, indicating that they are selectively regulating different G proteins within the same cell. Eliminating EGL-10 function in a *goa-1* null background has no additional phenotypic effect, suggesting that EGL-10 regulates $G_o\alpha$ (Koelle and Horvitz 1996). Our results indicate that EAT-16 does not regulate $G_o\alpha$ activity but instead regulates $G_q\alpha$ activity. Previous in vitro experiments on RGS proteins have provided some evidence that RGS proteins can act on G_q (Heximer et al. 1997; Zhang et al. 1998), but most RGS proteins examined could also act on $G_{i/o}$ family members (Heximer et al. 1997; Zhang et al. 1998). Our results provide in vivo evidence that RGS7 homologs can distinguish among major families of Gα-subunits.

Negative regulation of G_q by G_o

Analysis of double mutants involving *goa-1* and *egl-30* indicates that $G_o\alpha$ and $G_q\alpha$ function antagonistically in *C. elegans*. Although it is possible that G_q and G_o antagonize each other by positively and negatively regulating a common target, such as intracellular calcium, the identification of genes required for $G_o\alpha$ signaling that negatively regulate $G_q\alpha$ signaling argues that $G_o\alpha$ regulates behavior by modulating $G_q\alpha$ activity. Because we identified only one gene (*eat-16*) apparently upstream of *egl-30* in a fairly extensive screen for downstream targets of GOA-1, the number of steps between G_o and the G_q pathway might be small. In that view, G_o might antagonize G_q directly or might antagonize a downstream target of G_q, perhaps via SAG-1. A third possibility is that G_o antagonizes G_q signaling via EAT-16, in which case EAT-16 might be a direct effector for $G_o\alpha$ as well as being an RGS for $G_q\alpha$. G_o could modulate the activity of other Gα-subunits by activating one or more RGS proteins such as EAT-16 that in turn could down-regulate other Gα-subunits.

The data presented here suggest a model for the functions of GOA-1, EGL-30, EAT-16, and SAG-1 (Fig. 7). GOA-1 negatively regulates EGL-30 activity, possibly via EAT-16 or SAG-1. EGL-10 selectively regulates GOA-1 activity, whereas EAT-16 selectively regulates EGL-30 activity. EGL-30 has been shown to activate PLCβ in COS-7 cells (Brundage et al. 1996); therefore, second messengers generated from stimulation of PLCβ by $G_q\alpha$ are probably produced downstream of EGL-30. Reducing EAT-16 function would result in elevated levels of active EGL-30 subunits, which would in turn result in higher levels of second messengers and increased mobilization of internal calcium stores (Berridge 1993). SAG-1 negatively regulates the EGL-30 pathway. Whereas moderate overexpression of wild-type EGL-30 has been shown to cause hyperactive egg-laying and locomotion behaviors (Brundage et al. 1996), more intense overexpression of EGL-30 results in lethality (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.). We have shown that the *eat-16(sy438); sag-1(sy428)* double mutant is also inviable. The synthetic lethality of *sag-1* and *eat-16* mutations, as well as the lethality caused by expressing multiple copies of EGL-30, could be due to an excessive production of second messengers and/or excessive calcium release downstream of activated EGL-30. Reducing EGL-30 activity (and hence the level of downstream signaling) restores viability to *eat-16; sag-1* mutants.

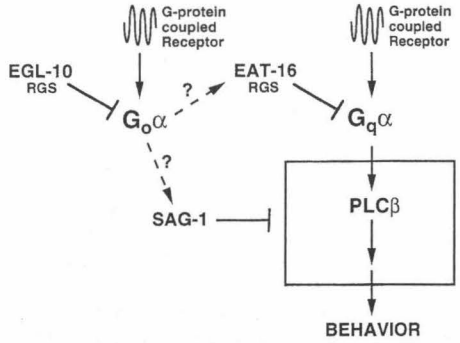


Figure 7. A model for regulation of behavior by GOA-1, EAT-16, and SAG-1. $G_o\alpha$ regulates behavior by antagonizing EGL-30-mediated signaling, either via EAT-16 and/or SAG-1 or an unknown effector. SAG-1 and EAT-16 function downstream, or parallel to, $G_o\alpha$ and operate synergistically to negatively regulate $G_q\alpha$ signaling. EAT-16 regulates EGL-30 by accelerating EGL-30 GTPase activity. SAG-1 also negatively regulates EGL-30 signaling and likely functions downstream of EGL-30, based on the stronger suppression of *egl-30* hypomorphs by *sag-1(sy428)*.

Behavior is modulated through a network of G proteins

Our results are consistent with a model in which a network of G protein pathways within cells can affect behavior by both positive and negative cross talk. Although synergistic effects between $G_{i/o}$ and G_q pathways have been observed (for review, see Selbie and Hill 1998), our results indicate negative regulation of $G_q\alpha$ or its downstream targets by $G_o\alpha$. That G_o and G_q function antagonistically in some way was implied from the opposite phenotypes of *goa-1* and *egl-30* mutations (Brundage et al. 1996). The isolation and analysis of GOA-1 suppressors involved in $G_q\alpha$ signaling support the model that $G_o\alpha$ functions to modulate behavior by down-regulating the G_q pathway in *C. elegans* and perhaps in other species as well. These results are analogous to the stimulatory and inhibitory effects of G_s and G_i on adenylyl cyclase (Hepler and Gilman 1992), raising the possibility that antagonistically acting G protein subunits are more universal than previously thought.

Materials and methods

Nematodes were cultured and handled according to standard procedures (Brenner 1974). All experiments were performed at 20°C except where otherwise noted. The following mutations and strains were used in this study for mapping experiments and double-mutant constructions: LGI *egl-30(ad805)*, *egl-30(ad809)*, DA1096 *egl-30(ad810)/szT1 [lon-2(e678)]* (Brundage et al. 1996), *egl-30(md186)*, *egl-30(n686)*, *unc-55(e402)*, MT363 *goa-1(n363)* (Ségalat et al. 1995), *dpy-5(e61)*, *unc-29(e1072)*, SP1726 *unc-29(h1) hP6 dpy-24(s71)* (a gift from J.A. Powell-Coffman, Iowa State University, Ames, IA), *mec-8(e398)*, *lin-11(n566)*, JK1553 *ces-1(n703d) qDf9/unc-29(e1702) lin-11(n566)* (Ellis and Kimble 1995). LGII: *unc-4(e120)*. LGIII: *unc-32(e189)*. LGIV: *dpy-20(e1282ts)*, PS1681 *dpy-20 syIs17[hsp::goa-1(Q205L)]* (Mendel et al. 1995), *unc-31(e169)*. LGV: *unc-42(e270)*, *him-5(e1490)*. LGX: TY2137 *meDf6; yDp13* (Akerib and Meyer 1994), PS1104 *egl-17(e1313) sli-1(sy143) unc-1*(e719), *dpy-3(e27)*, *unc-20(e112)*, *lin-15(n765ts)*. Linkage unknown: *syIs9[goa-1(Q205L)]* (Mendel et al. 1995), *syIs36[egl-30(+)]* (L. Brundage, P.W. Sternberg, and M.I. Simon, unpubl.).

Genetic screen

dpy-20(e1282) syIs17[hsp::goa-1(Q205L)] animals (Mendel et al. 1995) were mutagenized with ethylmethanesulfonate (21,000 haploid genomes) or trimethylpsoralen + UV irradiation (Yandell et al. 1994; 11,000 haploid genomes). F_2 progeny were heat-shocked (33°C, 30 min) as adults; moving animals were selected the next morning. All suppressors were backcrossed three times to the *syIs17* parent strain, with the suppression of heat shock-induced lethargy used as the criterion for scoring; the reference alleles were then outcrossed to N2 for characterization by selecting for the empty uterus and pale, scrawny appearance. *eat-16(sy438)* was originally isolated with another linked mutation that was removed by recombination during mapping experiments.

Characterization of mutants and double-mutant strains

To characterize the egg laying phenotype, animals were examined 24–28 hr after selecting them as L4 larvae, except for *syIs9[goa-1(Q205L)]* and *egl-30(n686)* strains, which were selected from mixed stage plates as gravid young adults before excess egg retention. A large number of staged adults were placed on a plate with *Escherichia coli* OP50. Newly laid eggs were harvested every 10–20 min and examined at 125× or with Nomarski optics (for *syIs9* strains). Cells in premature or wild-type eggs could be easily counted at this magnification. Later stage eggs were categorized qualitatively as follows: 20–50 cells (2–3 hr after fertilization), ~50 cells, precomma (gastrulation is beginning, before comma stage), comma (~5 hr after fertilization), twofold (~7 hr after fertilization), threefold (~9 hr after fertilization), and about to hatch. Eggs were considered premature if they contained eight or fewer cells. To count the number of eggs in the uterus, adults were examined at 125× magnification 24 hr after selecting as L4 larvae. N2, *eat-16/+*, *syIs9*, and Egl strains were bleached (as in Koelle and Horvitz 1996) to facilitate counting eggs. Hyperactive mutants were examined without bleaching.

To calculate pharyngeal pumps per minute, similarly staged adults were placed on individual plates seeded with OP50 and left undisturbed at least 15 min before counting. Pharyngeal pumps were counted for 2 min by pressing a counter once every three pumps; then, the numbers were multiplied by 1.5 to yield pumps per minute.

To calculate forward locomotion rate, staged adult animals were observed under conditions that maximize forward locomotion and minimize other behaviors (J. Mendel, pers. comm.): Two hundred microliters of a 5-ml OP50 culture was spread over the entire surface of a fresh 60-mm NGM plate preincubated at 20°C. Plates were left uncovered for the lawns to dry. After drying (which generally took ~1 hr), the plates were stored with lids on and used within 2 hr after drying. The result was a very thin lawn that covered the entire plate. Animals were left undisturbed on the lawns at least 5 min and then observed for 2 min. Seconds elapsed per sine wave (counting anterior flexing just posterior to the pharynx) were recorded using software written for this purpose by Hou-Pu Chou and Chieh Chang. Only forward flexing was counted, and waves right before or after a reversal were not included. Entries for all animals were then converted to waves/second and averaged. Averages and standard deviations were multiplied by 60 to yield waves per minute.

Because the presence of excess eggs in the uterus might affect locomotion rate, *egl-30* and *syIs9* strains were not staged as above; instead, young gravid adults with a single row of eggs in the uterus were selected from mixed-stage plates. Because of *syIs9* animals' tendency to travel in a circular manner (J. Mendel, unpubl.), one side of the body would often make a more visible flexion and the other side would not flex much, if at all; counting was done using the side that made the deeper flexions. Occasional animals did not move normally and may have been harmed during transfer; these animals' data were not included in the totals.

Characterization of sag-1/meDf6

meDf6; yDp13 males were mated to *unc-4(e120); sag-1(sy428) dpy-3(e27)* hermaphrodites and all nonUnc cross-progeny were selected as L4 larvae and examined 24 hr later. Cross-progeny were either non-Dpy or Dpy. *meDf6* deletes *sag-1* and *dpy-3*, and *yDp13* likely covers *sag-1* as well as *dpy-3*; therefore, Dpy progeny were assumed to be *sag-1 dpy-3/meDf6*, and Dp-bearing non-Dpy animals were examined in parallel as a control. All 16 *meDf6/sag-1(sy428) dpy-3(e27)* heterozygotes examined had empty uteri, and at least 15 of them suppressed the *syIs17[hs-G_oQL]* lethargy (the sixteenth animal crawled off the plate after heat shock treatment and could not be scored).

Mapping experiments

sag-1(sy428) was mapped between *egl-17* and *unc-1* by selecting Egl non-Unc recombinant progeny from *sy428/egl-17(e1313) sli-1(sy143) unc-1(e719)* heterozygous animals; 14 of 16 recombinants carried the *sy428* mutation. The following three-factor crosses determined the map position of *eat-16(sy438)*. First, *eat-16* was mapped between *unc-29* and *lin-11* by selecting recombinants from *unc-29 lin-11/eat-16; dpy-20 syIs17* heterozygotes. Nine of 13 Lin non-Unc recombinants carried *sy438* (scored by heat shock). Then, *sy438* was mapped right of *mec-8* by selecting Eat non-Dpy recombinants from *dpy-5 eat-16/mec-8; dpy-20 syIs17* heterozygotes. One of seven recombinants carried *mec-8*. (Recombinants were scored for *sy438* by the hyperactive phenotype and by heat shock.) Finally, *sy438* was mapped right of *hP6* by building + *mec-8* + *eat-16* + */unc-29* + *hP6* + *dpy-24* heterozygotes and selecting for non-Mec recombinants with empty uteri. Unc progeny from these recombinants were homozygosed, and the presence of *hP6* was determined by PCR amplification (Williams et al. 1992) using a mixture of three primers, 618 Tc1 primer (Williams et al. 1992), and two primers designed by J.A. Powell-Coffman (pers. comm.): hP6 (5'-TAG-

ATTTTGATCGTCTTCG) and hP62 (5′-TGTCTCGCCTACGATCTGATATTGC). Two of 10 Eat non-Mec recombinants carried *hP6*.

Transformation rescue

Animals were microinjected according to standard protocols (Mello et al. 1991; Mello and Fire 1995). The *lin-15* rescuing plasmid pbLH98 at 50 ng/µl (Huang et al. 1994) was used as the coinjection marker for all rescue experiments. pBluescript was included as carrier DNA to bring total DNA concentrations to 150–200 ng/µl. Strains bearing the temperature-sensitive *lin-15(n765)* mutation were cultured at 15°C before injection; afterwards, they were cultured at 22°C–23°C for 4–5 days, and non-Muv transformants were selected. Rescue was scored after at least one generation by heat-shocking non-Muv animals and looking for no suppression. Y20E10, C16C2, and three other cosmids contained within Y20E10 were each injected at ~50 ng/µl into *eat-16(sy438); dpy-20 syIs17; lin-15(n765)* animals. Subclone pYH5 was injected at a concentration of 30 ng/µl, and pWJC5 was injected at a concentration of 25 ng/µl.

Sequencing of eat-16 *mutations*

A 3-kb genomic DNA fragment was amplified (Williams et al. 1992) from *ad702* mutant animals in three independent reactions and from *sy438* animals in 10 independent reactions using the *Expand* long-range PCR kit (Boehringer Mannheim) with the following primers (from 5′ to 3′): AGACAGCTTCGTCGTATGTCTCAC ("P1") and GCAGTGTTGGGTGGTTCGAGATTG ("P2"); the products from each strain were gel-purified (Qiagen) and pooled. The *ad702* fragment was amplified a second time with P2 and the nested primer TGTCGAGCTGATTGAGACACGCTG ('S1') in 10 independent reactions; the products were purified as above and pooled. For both strains, PCR fragments were cloned into pGEM vectors (Stratagene), and the entire predicted gene product was sequenced (Kretz et al. 1989) in two plasmids per strain. The point mutations were then confirmed in the second strand and in both strands of three additional plasmids for *sy438* and four additional plasmids for *ad702*. For *sa735* and *sa609* mutants, products amplified as above in three independent PCR reactions were gel-purified, pooled, and sequenced directly.

Sequencing of eat-16 *cDNA*

Full-length cDNA sequence of *eat-16* was obtained from clone yk356b3, kindly provided by Yuji Kohara. Phage clones were excised in vitro and amplified in SOLR cells (Maniatis et al. 1982). Purified phagemids were then sequenced by the primers used for sequencing the *eat-16* mutations and primers for the T3 and T7 promoters. The splicing pattern was obtained by comparing yk356b3 with wild-type *eat-16* genomic sequence obtained from the GenBank database.

GFP-tagged expression of eat-16

Genomic DNA fragments including the *eat-16* promoter region and some coding exons were cloned into GFP expression vectors provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu (pers. comm.). Reporter construct pGP16 contains the 7.4-kb *Apa*I–*Bam*HI fragment and fuses to GFP-coding sequences in the ninth coding exon of *eat-16*. Reporter construct pGR02 contains the same upstream sequence but fuses to GFP-coding sequences in the first coding exon of *eat-16*. Both constructs were injected at 80 ng/µl into *lin-15(n765)* animals along with the *lin-15* rescuing plasmid pL15EK at 50 ng/µl (Clark et al. 1994) as a coinjection marker.

Double-mutant constructions

egl-30 eat-16 linked double mutants were constructed as follows: *dpy-5 eat-16*/++ males were mated to *egl-30* hermaphrodites (or *egl-30/szT1* heterozygotes, in the case of *ad810*). Non-Egl F_1 progeny were picked individually and removed the following day to synchronize the F_2 progeny. Plates with Dpy F_2 progeny were saved, and Eat non-Dpy animals were selected based on the empty uterus phenotype (which was transitory in these recombinants due to the semidominance of the *egl-30* alleles; see Brundage et al. 1996). Egl non-Dpy F3 progeny were saved, and the presence of *eat-16(sy438)* was confirmed by mating with N2 males and reisolating Eat non-Egl F_2 recombinants from all of several F_1 cross-progeny.

In the case of *ad810*, two Eat non-Dpy recombinants segregated Eat, Eat Dpy and arrested larvae; no viable Egl progeny were seen. Recombinants were mated with *szT1* males to balance the lethal chromosome. A parallel comparison of the lethal progeny of *ad810 sy438/szT1* and *ad810/szT1* was done by placing 16 worms of each strain on a plate with a thin bacterial lawn and removing them the next day. Dead larvae on both plates were observed over the course of several days and appeared similar.

Construction of double mutants between unlinked genes was straightforward; the strains were all confirmed either by complementation tests or by crossing with N2 males and reisolating both mutations. *egl-30(ad805) goa-1(n363); him-5(e1490)* was built by L. Brundage.

Transgenic strains

Strains containing *eat-16* transgenes were constructed by following the marker *lin-15(n765)* for *syEx256*, whereas G_o and G_q transgenes were followed by using *dpy-20(e1282)* as a rescuing marker. For example, a cross between *dpy-20* males and *dpy-20; lin-15; syEx256* hermaphrodites was kept for one day at 20°C to allow mating to occur and then cultured at 15°C to reduce the severity of the Dpy phenotype of the F_1 progeny. The resulting *dpy-20; lin-15; syEx256* males were mated to *dpy-20; syIs36; lin-15* hermaphrodites at 20°C, and non-Lin progeny were saved. The *goa-1(n363); syEx256* strain was constructed by mating *dpy-20/+; lin-15; syEx256* males to *goa-1(n363); lin-15* hermaphrodites and saving non-Dpy, non-Muv transgenic F_2 animals whose Lin (i.e., nontransgenic) progeny were all homozygous for *goa-1(n363)*. To test animals expressing both G_o and G_q transgenes, *dpy-20(e1282) syIs17* males were mated to *dpy-20(e1282); syIs36* hermaphrodites, and the resulting male progeny were heat-shocked.

Synthetic lethality of eat-16 *and* sag-1

To build the *eat-16; sag-1* double mutant, *mec-8 lin-11*/++; *sag-1* males were mated to *dpy-5 eat-16* hermaphrodites. Non-Dpy F_1 progeny were picked to individual plates, and homozygous Sag F_2s were picked from plates with Mec Lin progeny. To score penetrance of the lethality, 20 *dpy-5 + eat-16+/+mec-8 + lin-11; sag-1* L4 heterozygotes were placed on individual plates and transferred daily for 4 days. The total number of progeny was counted at the L4-Adult stage, yielding 2193 heterozygous, 936 Mec, and 52 Dpy animals. Dpy animals were saved and followed for a generation to determine their genotype.

Of the 50 viable Dpy animals, 48 were either *dpy++/dpy mec lin* or *dpy++/++lin* recombinants, one was a spontaneous male whose genotype could not be determined, and one escaped to adulthood and produced a few inviable progeny, a 0.1% survival rate. Arrested larvae were seen among the progeny of all 20 heterozygous mothers.

egl-30 eat-16; sag-1 *triple-mutant construction*

+eat-16+/mec-8 + lin-11; him-5; sag-1 males were mated to *egl-30(md186) eat-16* hermaphrodites, and F_1 progeny with empty uteri were picked to individual plates. From two plates that segregated Eat, Egl, and dead larvae but no Mec Lin (i.e., *egl eat/+ eat; sag-1/+*), 26 Egl F_2 progeny were picked to individual plates. None of the Egls produced lethal progeny. Seven of 26 Egls gave only Egl-30-like progeny, but on 18 plates, about one-fourth of the progeny were active, and one plate had only active progeny. Homozygosity of *sag-1* was confirmed by mating *dpy-20 syIs17* males with the triple mutant; male progeny were heat-shocked, and all were active 8 hr following heat shock ($n = 36$). As a control, *egl-30(md186) eat-16(sy438)* animals were also crossed to *syIs17* males; the male progeny resulting from this cross were lethargic 8 hr following heat shock ($n = 20$). Homozygosity of *eat-16* was confirmed by sequencing across the portion of the *eat-16* locus containing the *sy438* mutation in both strands, using a protocol similar to that described above.

COS-7 cell transfection and IP_3 assay

A 2.4-kb *Xho*I–*Xba*I fragment from yk356b3 (EAT-16 cDNA) was cloned into the pCIS vector to make pWJC4 for COS-7 cell transfection. M1 receptor (Bo Yu, pers. comm.) and EGL-30 (L. Brundage, pers. comm.) were also cloned in the same vector. Cells were transfected as described (Wu et al. 1992; Liu and Simon 1996) and seeded (1×10^5 per well) to 12-well plates the night before transfection. The pCIS vector was used as carrier DNA to normalize the total concentration of DNA in each well to 1.0 µg (for M1 receptor experiments) or 2.0 µg (for EGL-30 transfections). Lipofectin (5 µl) was added to each well. M1 receptor was activated by Carbachol (1 µM), and IP_3 assays were done as described (Liu and Simon 1996). Each transfection was performed in duplicate.

Acknowledgments

We thank Jane Mendel for the locomotion assay conditions; Hou-Pu Chou and Chieh Chang for writing software used to record locomotion data; John DeModena for technical assistance; Yuji Kohara for cDNA; Andrew Fire, Joohong Ahnn, Geraldine Seydoux, and Siqun Xu for GFP vectors; Merrilee Robatzek and James Thomas for *sa609* and *sa735*; Bo Yu, Jennifer Gu, and Valeria Mancino for assistance with COS-7 cell assays; Lorna Brundage, Carol Bastiani, Stephen Nurrish, Laurant Ségalat, Joshua Kaplan, Ron Ellis, and Jo Anne Powell-Coffman for sharing strains and communicating unpublished results; and Lorna Brundage, L. René Garcia, Jane Mendel, Carol Bastiani, Maureen Barr, Nadeem Moghal, Henry Lester, Mel Simon, and members of our laboratories for stimulating discussions and critical reading of this manuscript. Some of the strains used in this study were provided by the Caenorhabditis Genetics Center. This project is supported by HHMI, with which P.W.S. is an investigator.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked 'advertisement' in accordance with 18 USC section 1734 solely to indicate this fact.

References

Akerib, C.C. and B.J. Meyer. 1994. Identification of X chromosome regions in *Caenorhabditis elegans* that contain sex-determination signal elements. *Genetics* **138:** 1105–1125.

Aroian, R.V., A.D. Levy, M. Koga, Y. Ohshima, J.M. Kramer, and P.W. Sternberg. 1993. Splicing in *Caeonorhabditis elegans* does not require an AG at the 3′ splice acceptor site. *Mol. Cell. Biol.* **13:** 626–637.

Arshavsky, V.Y. and E.N. Pugh Jr. 1998. Lifetime regulation of G protein-effector complex: Emerging importance of RGS proteins. *Neuron* **20:** 11–14.

Avery, L. 1993. The genetics of feeding in *Caenorhabditis elegans. Genetics* **133:** 897–917.

Berman, D.M. and A.G. Gilman. 1998. Mammalian RGS proteins: Barbarians at the gate. *J. Biol. Chem.* **273:** 1269–1272.

Berman, D.M., T. Kozasa, and A.G. Gilman. 1996a. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem.* **271:** 27209–27212.

Berman, D.M., T.M. Wilkie, and A.G. Gilman. 1996b. GAIP and RGS4 are GTPase-Activating Proteins for the Gi subfamily of G protein α subunits. *Cell* **88:** 445–452.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature* **361:** 315–325.

Berstein, G., J.L. Blank, D.Y. Jhon, J.H. Exton, S.G. Rhee, and E.M. Ross. 1992. Phospholipase C-beta 1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell* **70:** 411–418.

Birnbaumer, L. 1992. Receptor-to-effector signaling through G proteins: Roles for beta gamma dimers as well as alpha subunits. *Cell* **71:** 1069–1072.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1990. The GTPase superfamily: A conserved switch for diverse cell functions. *Nature* **348:** 125–132.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: Conserved structure and molecular mechanism. *Nature* **349:** 117–127.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans. Genetics* **77:** 71–94.

Brundage, L., L. Avery, A. Katz, U.J. Kim, J.E. Mendel, P.W. Sternberg, and M.I. Simon. 1996. Mutations in a *C. elegans* Gqalpha gene disrupt movement, egg laying, and viability. *Neuron* **16:** 999–1009.

C. elegans Sequencing Consortium. 1998. Genome sequence of the nematode *C. elegans*: A platform for investigating biology [published erratum appears in *Science* 1999 Jan 1; **283:** (5398)35]. *Science* **282:** 2012–2018.

Clark, S.G., X. Lu, and H.R. Horvitz. 1994. The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137:** 987–997.

Coulson, A., J. Sulston, S. Brenner, and J. Karn. 1986. Toward a physical map of the genome of the nematode *Caenorhabditis elegans. Proc. Natl. Acad. Sci.* **83:** 7821–7825.

Coulson, A., R. Waterston, J. Kiff, J. Sulston, and Y. Kohara. 1988. Genome linking with yeast artificial chromosomes. *Nature* **335:** 184–186.

De Vries, L., M. Mousli, A. Wurmser, and M.G. Farquhar. 1995. GAIP, a protein that specifically interacts with the trimeric G protein G alpha i3, is a member of a protein family with a highly conserved core domain. *Proc. Natl. Acad. Sci.* **92:** 11916–11920.

Dohlman, H.G., J. Song, D. Ma, W.E. Courchesne, and J. Thorner. 1996. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: Expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Mol. Cell. Biol.* **16:** 5194–5209.

Dohlman, H.G. and J. Thorner. 1997. RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* **272:** 3871–3874.

Ellis, R.E. and J. Kimble. 1995. The *fog-3* gene and regulation of cell fate in the germ line of *Caenorhabditis elegans*. *Genetics* **139:** 561–577.

Faurobert, E. and J.B. Hurley. 1997. The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin *in vitro*. *Proc. Natl. Acad. Sci.* **94:** 2945–2950.

Freyd, G., S.K. Kim, and H.R. Horvitz. 1990. Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene lin-11. *Nature* **344:** 876–879.

Graziano, M.P. and A.G. Gilman. 1989. Synthesis in *Escherichia coli* of GTPase-deficient mutants of Gs alpha. *J. Biol. Chem.* **264:** 15475–15482.

Hepler, J.R. and A.G. Gilman. 1992. G proteins. *Trends Biochem. Sci.* **17:** 383–387.

Heximer, S.P., N. Watson, M.E. Linder, K.J. Blumer, and J.R. Hepler. 1997. RGS2/G0S8 is a selective inhibitor of Gq alpha function. *Proc. Natl. Acad. Sci.* **94:** 14389–14393.

Hodgkin, M.N., T.R. Pettitt, A. Martin, R.H. Michell, A.J. Pemberton, and M.J. Wakelam. 1998. Diacylglycerols and phosphatidates: Which molecular species are intracellular messengers? *Trends Biochem. Sci.* **23:** 200–204.

Hsu, W.H., U. Rudolph, J. Sanford, P. Bertrand, J. Olate, C. Nelson, L.G. Moss, A.E. Boyd, J. Codina, and L. Birnbaumer. 1990. Molecular cloning of a novel splice variant of the alpha subunit of the mammalian Go protein. *J. Biol. Chem.* **265:** 11220–11226.

Huang, L.S., P. Tzou, and P.W. Sternberg. 1994. The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. *Mol. Biol. Cell* **5:** 395–411.

Hunt, T.W., T.A. Fields, P.J. Casey, and E.G. Peralta. 1996. RGS10 is a selective activator of Gai GTPase activity. *Nature* **383:** 175–177.

Koelle, M.R. 1997. A new family of G-protein regulators—the RGS proteins. *Curr. Opin. Cell Biol.* **9:** 143–147.

Koelle, M.R. and H.R. Horvitz. 1996. EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell* **84:** 115–125.

Korswagen, H.C., J.H. Park, Y. Ohshima, and R.H. Plasterk. 1997. An activating mutation in a *Caenorhabditis elegans* Gs protein induces neural degeneration. *Genes & Dev.* **11:** 1493–1503.

Kretz, K.A., G.S. Carson, and J.S. O'Brien. 1989. Direct sequencing from low-melt agarose with Sequenase [published erratum appears in *Nucleic Acids Res.* 1990. **18:** (2)400]. *Nucleic Acids Res.* **17:** 5864.

Lavu, S., J. Clark, R. Swarup, K. Matsushima, K. Paturu, J. Moss, and H.F. Kung. 1988. Molecular cloning and DNA sequence analysis of the human guanine nucleotide-binding protein Go alpha [published erratum appears in *Biochem. Biophys. Res. Commun.* 1988. **153:** (1)487]. *Biochem. Biophys. Res. Commun.* **150:** 811–815.

Liu, M. and M.I. Simon. 1996. Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. *Nature* **382:** 83–87.

Lochrie, M.A., J.E. Mendel, P.W. Sternberg, and M.I. Simon. 1991. Homologous and unique G protein alpha subunits in the nematode *Caenorhabditis elegans*. *Cell Regul.* **2:** 135–154.

Lundquist, E.A. and R.K. Herman. 1994. The *mec-8* gene of *Caenorhabditis elegans* affects muscle and sensory neuron function and interacts with three other genes: *unc-52*, *smu-1* and *smu-2*. *Genetics* **138:** 83–101.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual*, Vol. 1, pp. 2.60–2.63. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mello, C. and A. Fire. 1995. DNA Transformation. In Caenorhabditis elegans: *Modern biological analysis of an organism* (ed. D.C. Shakes and H.F. Epstein), pp. 451–482. Academic Press, San Diego, CA.

Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10:** 3959–3970.

Mendel, J.E., H.C. Korswagen, K.S. Liu, Y.M. Hajdu-Cronin, M.I. Simon, R.H. Plasterk, and P.W. Sternberg. 1995. Participation of the protein Go in multiple aspects of behavior in *C. elegans*. *Science* **267:** 1652–1655.

Miller, K.G., A. Alfonso, M. Nguyen, J.A. Crowell, C.D. Johnson, and J.B. Rand. 1996. A genetic selection for *Caenorhabditis elegans* synaptic transmission mutants. *Proc. Natl. Acad. Sci.* **93:** 12593–12598.

Natochin, M. and N.O. Artemyev. 1998. A single mutation Asp229 → Ser confers upon Gs alpha the ability to interact with regulators of G protein signaling. *Biochemistry* **37:** 13776–13780.

Offermanns, S., T. Wieland, D. Homann, J. Sandmann, E. Bombien, K. Spicher, G. Schultz, and K.H. Jakobs. 1994. Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11. *Mol. Pharmacol.* **45:** 890–898.

Olate, J., H. Jorquera, P. Purcell, J. Codina, L. Birnbaumer, and J.E. Allende. 1989. Molecular cloning and sequence determination of a cDNA coding for the alpha-subunit of a Go-type protein of *Xenopus laevis* oocytes [published erratum appears in *FEBS Lett.* 1990 Jul 16; **267:** (2)316]. *FEBS Lett.* **244:** 188–192.

Ségalat, L., D.A. Elkes, and J.M. Kaplan. 1995. Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. *Science* **267:** 1648–1651.

Selbie, L.A. and S.J. Hill. 1998. G protein-coupled-receptor cross-talk: The fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol. Sci.* **19:** 87–93.

Simon, M.I., M.P. Strathmann, and N. Gautam. 1991. Diversity of G proteins in signal transduction. *Science* **252:** 802–808.

Snow, B.E., A.M. Krumins, G.M. Brothers, S.F. Lee, M.A. Wall, S. Chung, J. Mangion, S. Arya, A.G. Gilman, and D.P. Siderovski. 1998. A G protein gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. *Proc. Natl. Acad. Sci.* **95:** 13307–13312.

Sondek, J., D.G. Lambright, J.P. Noel, H.E. Hamm, and P.B. Sigler. 1994. GTPase mechanism of G proteins from the 1.7-A crystal structure of transducin alpha-GDP-AIF-4. *Nature* **372:** 276–279.

Starr, T., A.M. Howell, J. McDowall, K. Peters, and A.M. Rose. 1989. Isolation and mapping of DNA probes within the linkage group I gene cluster of *Caenorhabditis elegans*. *Genome* **32:** 365–372.

Sternweis, P.C. and J.D. Robishaw. 1984. Isolation of two proteins with high affinity for guanine nucleotides from mem-

branes of bovine brain. *J. Biol. Chem.* **259:** 13806–13813.

Sulston, J., Z. Du, K. Thomas, R. Wilson, L. Hillier, R. Staden, N. Halloran, P. Green, J. Thierry-Mieg, L. Qiu et al. 1992. The *C. elegans* genome sequencing project: A beginning. *Nature* **356:** 37–41.

Tesmer, J.J., D.M. Berman, A.G. Gilman, and S.R. Sprang. 1997. Structure of RGS4 bound to AIF4–activated Gia1: Stabilization of the transition state for GTP hydrolysis. *Cell* **89:** 251–261.

Trent, C., N. Tsung, and H.R. Horvitz. 1983. Egg-laying defective mutants of the nematode *Caenorhabditis elegans. Genetics* **104:** 619–647.

van der Voorn, L., M. Gebbink, R.H.A. Plasterk, and H.L. Ploegh. 1990. Characterization of a G-protein b-subunit gene from the *Caenorhabditis elegans. J. Mol. Biol.* **213:** 17–26.

Van Meurs, K.P., C.W. Angus, S. Lavu, H.F. Kung, S.K. Czarnecki, J. Moss, and M. Vaughan. 1987. Deduced amino acid sequence of bovine retinal Go alpha: Similarities to other guanine nucleotide-binding proteins. *Proc. Natl. Acad. Sci.* **84:** 3107–3111.

Watson, N., M.E. Linder, K.M. Druey, J.H. Kehrl, and K.J. Blumer. 1996. RGS family members: GTPase-activating proteins for heterotrimeric G-proteins a-subunits. *Nature* **383:** 172–175.

Williams, B.D., B. Schrank, C. Huynh, R. Shownkeen, and R.H. Waterston. 1992. A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* **131:** 609–624.

Wu, D.Q., C.H. Lee, S.G. Rhee, and M.I. Simon. 1992. Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J. Biol. Chem.* **267:** 1811–1817.

Yandell, M.D., L.G. Edgar, and W.B. Wood. 1994. Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans. Proc. Natl. Acad. Sci.* **91:** 1381–1385.

Yoon, J., R.D. Shortridge, B.T. Bloomquist, S. Schneuwly, M.H. Perdew, and W.L. Pak. 1989. Molecular characterization of *Drosophila* gene encoding Go alpha subunit homolog. *J. Biol. Chem.* **264:** 18536–18543.

Zhang, S., N. Watson, J. Zahner, J.N. Rottman, K.J. Blumer, and A.J. Muslin. 1998. RGS3 and RGS4 are GTPase activating proteins in the heart. *J. Mol. Cell. Cardiol.* **30:** 269–276.

Chapter 4:

Reconstitute *C. elegans* G protein signaling in mammalian cell culture

Abstract:

The results of this chapter is based on the reconstitution experiments done in mammalian cell culture to answer the questions that are hard to address by *C. elegans* genetics. Through transcient transfection of *C. elegans* genes to mammalian cell lines and assay PLC activity mediated by Gq/G11, we studied function of RGS protein EAT-16 on Gq. The RGS domain of EAT-16 is required for its function as a negative regulator of Gq. The *C. elegans* Gβ5 homologue GPB-2 interacts with EAT-16 in vitro and it negatively regulates Gq/G11, suggesting GPB-2 might associate with EAT-16 and facilitate its action. *C. elegans* Go alpha subunit GOA-1 also acts as a strong inhibitor of Gq/G11, favoring the model that Gq pathway is downstream of Go. The Gq pathway may be negatively regulated by GOA-1 through Go effectors such as EAT-16 or GPB-2.

Introduction:

C. elegans Go and Gq pathways regulate locomotion and egg-laying behaviors in a opposite way. Mutant animals with loss-of-function *goa-1* or gain-of-function *egl-30* are hyperactive and egg-laying constitutive, while both *goa-1(xs)* and *egl-30 (lf)* cause animals to be lethargic and egg-laying defective (Brundage et al., 1996, Mendel et al., 1995, Segalat et al., 1995). Two RGS7 homologues were identified in *C. elegans* regulating egg-laying and locomotion. *egl-10* is the RGS protein for *goa-1* (Koelle and Horvitz, 1996) and *egl-10 (lf)* animals are egg-laying defective and lethargic. *eat-16* is the RGS protein for *egl-30* (Hajdu-Cronin et al., 1999) and *eat-16 (lf)* animals are hyperactive and egg-laying constitutive. The molecular nature of these two RGS proteins that causes G protein specificity has not been found.

People have realized that Go and Gq pathways antagonize each other to regulate same behaviors. Epistatic assays done between *goa-1* and *egl-30* mutants indicate that Gq is more epistatic to Go, although intermediate phenotypes were always observed (Hajdu-Cronin et al., 1999, Miller et al., 1999). It is difficult to prove the actual relationship between the two pathways. The parallel model proposes that Go and Gq pathways

function in parallel to regulate locomotion and egg-laying. According to this model, the epistatic effect is because Gq signaling is more dominant than Go signaling. However linear model proposes that Go acts upstream of Gq. It is natural to explain the fact Gq is epistatic because *egl-30* is downstream of *goa-1* in this model. To distinguish these two models, we studied these proteins in a reconstituted system.

COS-7 cells have high level of endogenous Gq/G11, most RGS proteins and other G protein subunits. It also has endogenous M1 receptor (Thelander and Berg, 1986), which couples to Gq/G11 (Ashkenazi et al., 1989, Gutkind et al., 1991, Stephens et al., 1993). Assays on Gq/G11 induced PLC activity were developed to study functions of other genes on G protein signaling (Liu and Simon, 1996, Wu et al., 1992).

In this paper, we partially reconstituted the *C. elegans* G-protein signaling network in mammalian cell culture and studied the function of RGS protein EAT-16 on mammalian Gq/G11. We realized that studying the activity of a intermediate product downstream of Gq/G11 signaling is the key to solve the question whether Go act upstream of Gq. However, this approach cannot be easily done by *C. elegans* genetics. We took the advantage of cell culture assays to study the activity of direct effector of Gq/G11 signaling: PLCβ. By measuring PLC activity induced by M1 receptor and Gq/G11, we were able to study the effect of other genes on Gq/G11 pathway. EAT-16 acts as a negative regulator for Gq signaling, and deleting its RGS domain abolishes the function. Transfecting GOA-1 to the system causes decrease of PLC activity, suggests that Go is able to inhibit Gq signaling when they are in the same cell. It supports the model that Go and Gq functions linearly. Since RGS7 proteins are known to interact with Gβ5 (Siderovski et al., 1999) and they are co-expressed (Kovoor et al., 2000), we studied the effect of Gβ5 in the system. *C. elegans* Gβ5 gene interacts with EAT-16 in vitro (C. Bastiani and P. Snow, pers. comm.). GPB-2 also decrease PLC activity induced by Gq, suggesting it may associate with and help the GAP activity of RGS proteins. Through above results, we propose the linear pathway that Go negatively regulates Gq, possibly through Gβ5 and RGS proteins.

Results:

EAT-16 negatively regulates PLC activity induced by Gq

Previously we showed that EAT-16 negatively regulate PLC activity in COS-7 cells (Hajdu-Cronin et al., 1999). The plasmid pWJC4 contains a full-length cDNA of EAT-16 under pCMV promoter. Transfection of pWJC4 to COS-7 cells decrease PLC activity comparing with control vector DNA **(Figure 1A)**. Similar results were obtained when we transfected the same plasmid into a cell line lacking endogenous Gq/G11 (data not shown). pWJC4 negatively regulate PLC activity induced by M1 receptor and EGL-30 in the Gq-G11- cell line (data not shown). Both experiments indicated that EAT-16 function as the RGS protein for Gq.

To furthur study the function of EAT-16, we constructed a series of plasmid containing different truncations of EAT-16 in the pCDNA3.1(+) vector. To facilitate future studies, we HA-tagged EAT-16 in all these constructs. The HA-tagged full-length EAT-16 construct pWJC8 works as well as previously used pWJC4 **(Figure 1B),** which contains full-length EAT-16 in pCIS vector (Hajdu-Cronin et al., 1999). All these results are consistent with the genetics data that *eat-16* acts as the RGS protein for *egl-30*, which encodes the *C. elegans* Gq.

The RGS domain of EAT-16 is required for its function

The construct pWJC7 only has the N-terminal DEP domain of EAT-16. Transfection of pWJC7 does not significantly reduce PLC activity **(Figure 2)**. While the construct pWJC9 has both GGL domain and RGS domain, and it works as well as pWJC8 during the test **(Figure 2)**. These results indicates deleting GGL domain and RGS domain from EAT-16 eliminate its function as a negative regulator of Gq/G11, but loss of DEP domain does not affect EAT-16 function in the cell. This is consistent with previous model the EAT-16 functions as the GAP of Gq/G11 and its RGS domain is essential for the GAP activity.

GPB-2 negatively regulate PLC activity induced by Gq/G11

Since EAT-16 has the Gγ like GGL domain and there is a *C. elegans* Gβ5 homologue GPB-2, we speculated the possibility that EAT-16 may interact with GPB-2 to carry out its RGS function. Recombinant EAT-16 protein and GPB-2 protein interacts with each other in vitro (C. Bastiani and P. Snow, pers. comm.). We transfected GPB-2 cDNA in pcDNA3.1(+) vector (C. Bastiani, pers. comm.) into COS-7 cells and found it also decrease PLC activity mediated by Gq/G11, and the effect is dependent on the dosage of GPB-2 transfected **(Figure 3)**. These data suggests that GPB-2 is a negative regulator of Gq. The mechanism of the inhibition we observed might be related to endogenous RGS proteins in the cells.

GOA-1 negatively regulate PLC activity induced by Gq/G11

The question raised by previous study about whether Go and Gq pathway functions linearly or in parallel is very hard to distinguish through genetic approach. To find out the effect of Go on Gq signaling, we measured PLC activity induced by Gq/G11 in the presence of overexpressed GOA-1. Transfecting GOA-1 cDNA into COS-7 cells indicates Gq/G11 mediated PLC activity decrease under the effect of GOA-1. The inhibition by GOA-1 is not due to the effect of βγ since a similar result were obtained both by wild type GOA-1 **(Figure 4A)** and constitutively activated GOA-1 **(Figure 4B)** which does not interact with βγ.

Discussion:

The results we obtained from mammalian cell culture fully support the model based on our genetic data (Hajdu-Cronin et al., 1999). The RGS protein *eat-16* was proposed as the GAP specific for *egl-30* (Gq). We demonstrated it in mammalian cells that EAT-16 is a negative regulator for mammalian Gq/G11 and *C. elegans* Gq EGL-30. Then we studied which domains in EAT-16 conduct the activity. As it is known that RGS domain is required for the GAP activities of RGS proteins, our result is consistent with it. Truncation version of EAT-16 without DEP domain works as well as full length EAT-16, and truncation version of EAT-16 without GGL and RGS domain lost its activity as a

negative regulator of Gq/G11. The fact that pWJC7 does not lead to decrease of PLC activity indicates that negative regulations we observed when using other constructs are not due to non-specific effect on transfection or assay.

The second question we wanted to address in this paper is the function of GPB-2 as a Gβ5 homologue. It has been reported several times that Gβ5 interacts with long RGS such as RGS6, 7, 9 and 11(Siderovski et al., 1999) and Gβ5 co-express with RGS7 (Kovoor et al., 2000). Recombinant GPB-2and EAT-16 proteins interact with each other in vitro. The result that GPB-2 also acts as a negative regulator of Gq/G11 indicates GPB-2 may help endogenous RGS proteins in COS-7 cells to perform their GAP activity towards Gq/G11. Furthur biochemical binding assays will be very necessary to tell whether they do bind each other in the mammalian cells and in *C. elegans*.

The most important answer we wanted to find out from this study is the function of Go, since it has been a mystery since its discovery. It is surprising that in a screen for suppressors of activated Goα, we identified two genes in Gq signaling, *dgk-1* and *eat-16*. How do these two genes relate to Go was a puzzle. There are two models explaining the fact they are suppressor of activated Go. One is the parallel model: Go acts independently from Gq and they regulate the same behaviors antagonistically; *eat-16* and *dgk-1* are negative regulators of Gq signaling, thus in these mutants, up regulated Gq signaling overcome the opposite effects created by activated Go. The other model is the linear model: Go function upstream of Gq pathway; Go negatively regulate Gq through effectors; the link between Go and Gq may be *eat-16*, *dgk-1* or unidentified genes. These two models are very difficult to distinguish by genetic analysis, since the final output of behavior may be similar in both cases and genetics is based on the observation of phenotypes. The easiest way to tell them apart is by measuring the intermediate product of one of the signaling pathways, such as PLC activity. Due to the complication of structure of *C. elegans* and lack of worm cell culture, the most straightforward system we can use at present is mammalian cell culture. The receptors and endogenous Gq/G11 in COS-7 cells mimics what happens in *C. elegans* single cell.

The fact GOA-1 down regulate PLC activity induced by M1 receptor and Gq/G11 indicates that Go actually affect Gq signaling, which is consistent with the linear hypothesis that Go is upstream of Gq **(Figure 5)**. GOA-1 may negative regulate Gq/G11 induced PLC activity through endogenous RGS/Gβ5 in COS-7 cells. The steps between Go and Gq are still a mystery. Since genetically there has not been any mutant that only suppresses Go but not Gq, it is possible few elements are at this link. EAT-16 interacts with GPB-2 and there are possible interactions between GPB-2 with G protein alpha subunits, which makes EAT-16 and GPB-2 likely to be the Go effectors that negatively regulate Gq pathway.

Both models are built on the assumption that Go and Gq signaling function in the same cells in *C. elegans*. Although GFP expression and antibody staining experiments indicated that *goa-1*, *egl-30*, *egl-10* and *eat-16* have similar patterns of distribution in tissues, more stringent experiments such as mosaic analysis and phenotype rescue using tissue-specific promoters. Expressing recombinant proteins of these genes and in vitro assaying GAP activity and protein-protein interaction will be required to prove our genetic hypothesis.

Acknowlegement

The authur thank C. Bastiani for the GPB-2 construct and J. Gu for the Gq-G11- cell line. All cell culture transfections and assays were done in the Lab of M. I. Simon.

Materials and Methods.

Plasmid construction

pWJC4 contains full length cDNA of EAT-16. It is made by inserting 2.4 kb Xho1/XbaI fragment from cDNA clone yk356b3 into pcDNA3.1(+) vector.

pWJC7 contains truncated EAT-16 DEP domain with HA-tag. Construct was made by PCR amplifying from yk356b3 using primers S2P1 (5' CGCGAGCTCGA

GATGTACCCA TACGACGTCC CAGACTACGC TATGATGCCG TTGACCAAG) and S2P5 (5' GCGAGATCTA GAACCTTGTG CATTCTGCCG TCG). The 0.7 kb PCR fragment was digested by XhoI/XbaI, then insert into pcDNA3.1(+) vector.

pWJC8 contains full length EAT-16 cDNA with HA-tag. Construct was made by PCR amplifying from yk356b3 using primers S2P1 and S2P2 (5' GCGAGATCTA GAATGACGTC ATTCGGGATC CGG). The 1.3 kb PCR fragment was digested by XhoI/XbaI, then insert into pcDNA3.1(+) vector.

pWJC9 contains truncated EAT-16 GGL and RGS domain with HA-tag. Construct was made by PCR amplifying from yk356b3 using primers S2P2 and S2P5. The 0.9 kb PCR fragment was digested by XhoI/XbaI, then insert into pcDNA3.1(+) vector.

PWJC6 contains wild type GOA-1 cDNA. Construct was made by dropping 1.5 kb smaI/XhoI fragment from pCeGoα (Lochrie et al., 1991) into pcis vector.

pWJC10 contains activated GOA-1. Site directed mategenesis of GOA-1 was made by PCR using primer pair GoP1 (5' CGCGAGCTCG AGGGTCCACC GTTCATCAAC TC)/ GoP3 (5' GATGTGGGAG GTCTTAGATC AGAAAGG) and primer pair GoP2 (5' GCGAGATCTA GAGGGCGGCG AAGACGACAGC)/ GoQL (5' ACCGCGGTGG CTAGCGCTCT AGAAC), then combine the products and PCR amplify using primer pair GoP1/GoP2. The 1.5 kb fragment of secondary PCR was digested by XhoI/XhaI, then inserted into vector pcDNA3.1(+)

Plasmid GPB-2 contains full length cDNA of GPB-2 in pcDNA vector (C. Bastiani, unpublished data).

Transient transfection:

For Lipofectin transfection, cells are cultured and transfected as described before (Liu and Simon, 1996, Wu et al., 1992). Cells were seeded ($1x10^5$/well) to 12 well plates the night before transfection. Either pcis or pcDNA vector DNA (depending on the experiment) were used as carrier DNA to normalize the final concentration of DNA in each well to 1.0 μg. Every transfection sample contains 0.1 μg pcis-LacZ. Lipofectin were added at 5μl/well. All the transfections are done in quadrant, Two for β-gal assay and two for IP3 assay.

For Superfect transfection, standard protocol from Qiagen was used. $5x10^4$ cells were seeded to 12 well plates the day before transfection. 5 μl Superfect reagent was added to each well instead of Lipofectin. Cells were incubated in transfection mix at 37°C for 3 hours before they were switched to fresh 10% FBS. Either pcis or pcDNA vector DNA (depending on the experiment) were used as carrier DNA to normalize the final concentration of DNA in each well to 1.0 μg. Every transfection sample contains 0.1 μg pcis-LacZ. All the transfections are done in quadrant, Two for β-gal assay and two for IP3 assay.

β-gal assay and IP3 assay

36-48 hours after transfection, two transfected wells from the quadrant were assayed for b-gal activity following the micro β-Galactosidase Assay protocol from STRATAGENE. β-gal activity were calculated by measuring O.D. at 405nm. Average of the β-gal activity was used as the transfection efficiency. IP3 assays were done on the other two transfections on the quadrant as described before (Liu and Simon, 1996). M1 receptor was activated by Carbachol [1μM].

Data analysis

The output from IP3 assays was divided by transfection efficiency (obtained from β-gal assay). The result is used as the final result of PLC activity. Standard Deviation were calculated by INSTAT and plotted by Cricket Graph.

References:

Ashkenazi, A., Ramachandran, J. and Capon, D.J. (1989). Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. Nature *340*, 146-50.

Brundage, L., Avery, L., Katz, A., Kim, U.J., Mendel, J.E., Sternberg, P.W. and Simon, M.I. (1996). Mutations in a C. elegans Gqalpha gene disrupt movement, egg laying, and viability. Neuron *16*, 999-1009.

Gutkind, J.S., Novotny, E.A., Brann, M.R. and Robbins, K.C. (1991). Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. Proc Natl Acad Sci U S A *88*, 4703-7.

Hajdu-Cronin, Y.M., Chen, W.J., Patikoglou, G., Koelle, M.R. and Sternberg, P.W. (1999). Antagonism between G(o)alpha and G(q)alpha in Caenorhabditis elegans: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. Genes Dev *13*, 1780-93.

Koelle, M.R. and Horvitz, H.R. (1996). EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. Cell *84*, 115-25.

Kovoor, A., Chen, C.K., He, W., Wensel, T.G., Simon, M.I. and Lester, H.A. (2000). Co-expression of Gbeta5 enhances the function of two Ggamma subunit-like domain-containing regulators of G protein signaling proteins. J Biol Chem *275*, 3397-402.

Liu, M. and Simon, M.I. (1996). Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. Nature *382*, 83-7.

Lochrie, M.A., Mendel, J.E., Sternberg, P.W. and Simon, M.I. (1991). Homologous and unique G protein alpha subunits in the nematode Caenorhabditis elegans. Cell Regul *2*, 135-54.

Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin, Y.M., Simon, M.I., Plasterk, R.H. and Sternberg, P.W. (1995). Participation of the protein Go in multiple aspects of behavior in C. elegans. Science *267*, 1652-5.

Miller, K.G., Emerson, M.D. and Rand, J.B. (1999). Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in C. elegans. Neuron *24*, 323-33.

Segalat, L., Elkes, D.A. and Kaplan, J.M. (1995). Modulation of serotonin-controlled behaviors by Go in Caenorhabditis elegans. Science *267*, 1648-51.

Siderovski, D.P., Strockbine, B. and Behe, C.I. (1999). Whither goest the RGS proteins? Crit Rev Biochem Mol Biol *34*, 215-51.

Stephens, E.V., Kalinec, G., Brann, M.R. and Gutkind, J.S. (1993). Transforming G protein-coupled receptors transduce potent mitogenic signals in NIH 3T3 cells independent on cAMP inhibition or conventional protein kinase C. Oncogene *8*, 19-26.

Thelander, L. and Berg, P. (1986). Isolation and characterization of expressible cDNA clones encoding the M1 and M2 subunits of mouse ribonucleotide reductase. Mol Cell Biol *6*, 3433-42.

Wu, D.Q., Lee, C.H., Rhee, S.G. and Simon, M.I. (1992). Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. J Biol Chem *267*, 1811-7.

Figure legend:

Figure 1: A. EAT-16 negatively regulates PLC activity induced by M1 receptor and endogenous Gq/G11. Lipofectin transfection. Lane 1: pcis control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg pWJC4. B. HA tagged EAT-16 negatively regulates PLC activity induced by M1 receptor and endogenous Gq/G11. Superfect transfection. Lane 1: pcDNA control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg pWJC8.

Figure 2: pWJC7(DEP domain) does not affect PLC activity. pWJC9 (GGL and RGS domain) functions as well as the full length EAT-16 construct pWJC8 in negatively regulating PLC activity. Superfect transfection. Lane 1: pcDNA control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg pWJC7; Lane 4: 0.1 μg M1 with 0.4 μg pWJC8; Lane 5: 0.1 μg M1 with 0.4 μg pWJC9.

Figure 3: GPB-2 acts as a negative regulator of PLC activity induced by Gq/G11. Lipofectin transfection. Lane 1: pcDNA control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg GPB-2; Lane 3: 0.1 μg M1 with 0.8 μg GPB-2.

Figure 4: A. wild type GOA-1 construct pWJC6 negatively regulates PLC activity. Lipofectin transfection. Lane 1: pcis control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg pWJC6. B. Similar effect were seen when using constitutively activated GOA-1 with Q205->L mutation. Superfect transfection. Lane 1: pcDNA control; Lane 2: 0.1 μg M1; Lane 3: 0.1 μg M1 with 0.4 μg pWJC10.

Figure 5: Go negatively regulate Gq signaling: the linear model.

Figure 1

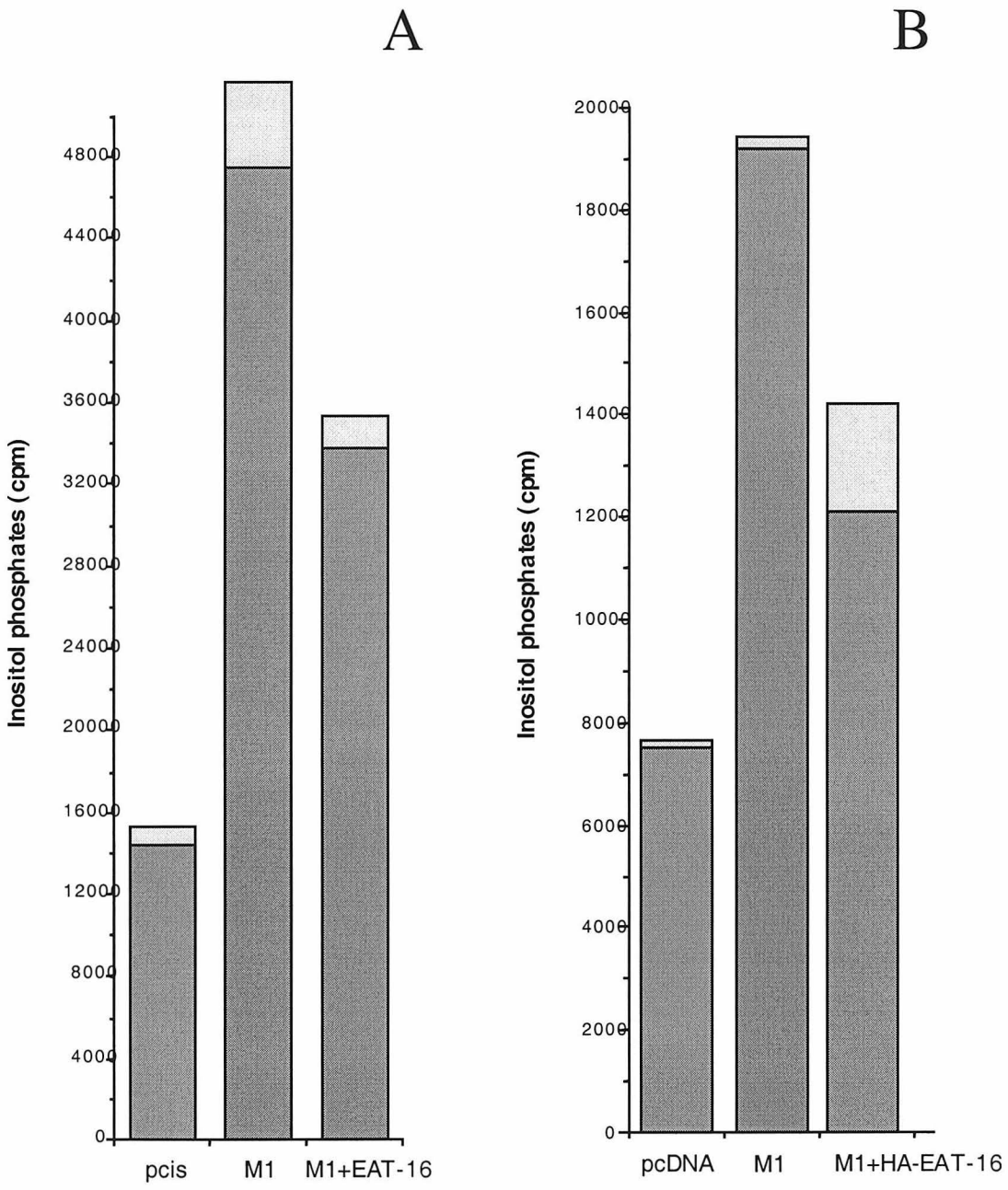

A
B
Inositol phosphates (cpm)
0
4000
8000
12000
16000
20000
24000
28000
32000
36000
40000
44000
48000
pcis
M1
M1+EAT-16
Inositol phosphates (cpm)
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
pcDNA
M1
M1+HA-EAT-16

Figure 2

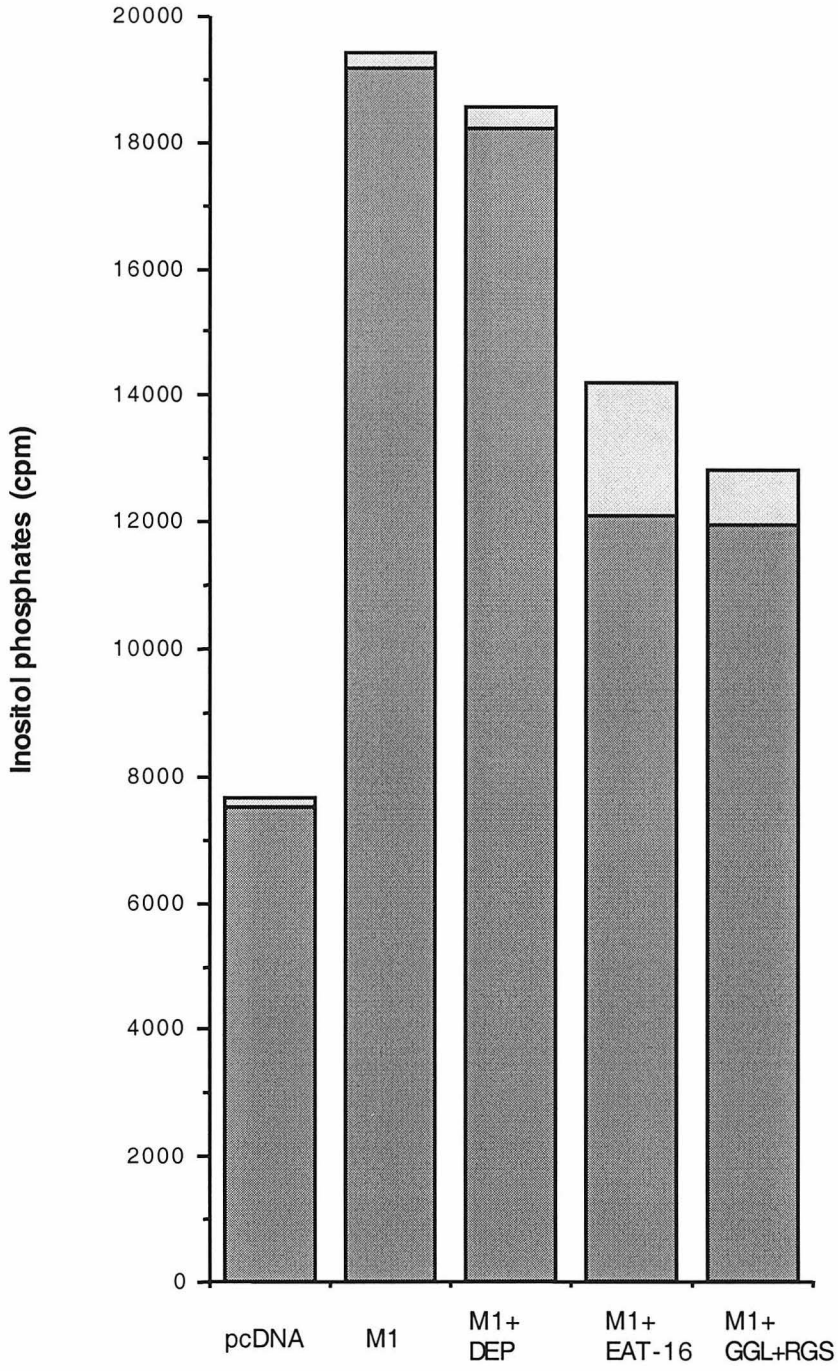

Inositol phosphates (cpm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
pcDNA
M1
M1+ DEP
M1+ EAT-16
M1+ GGL+RGS

Figure 3

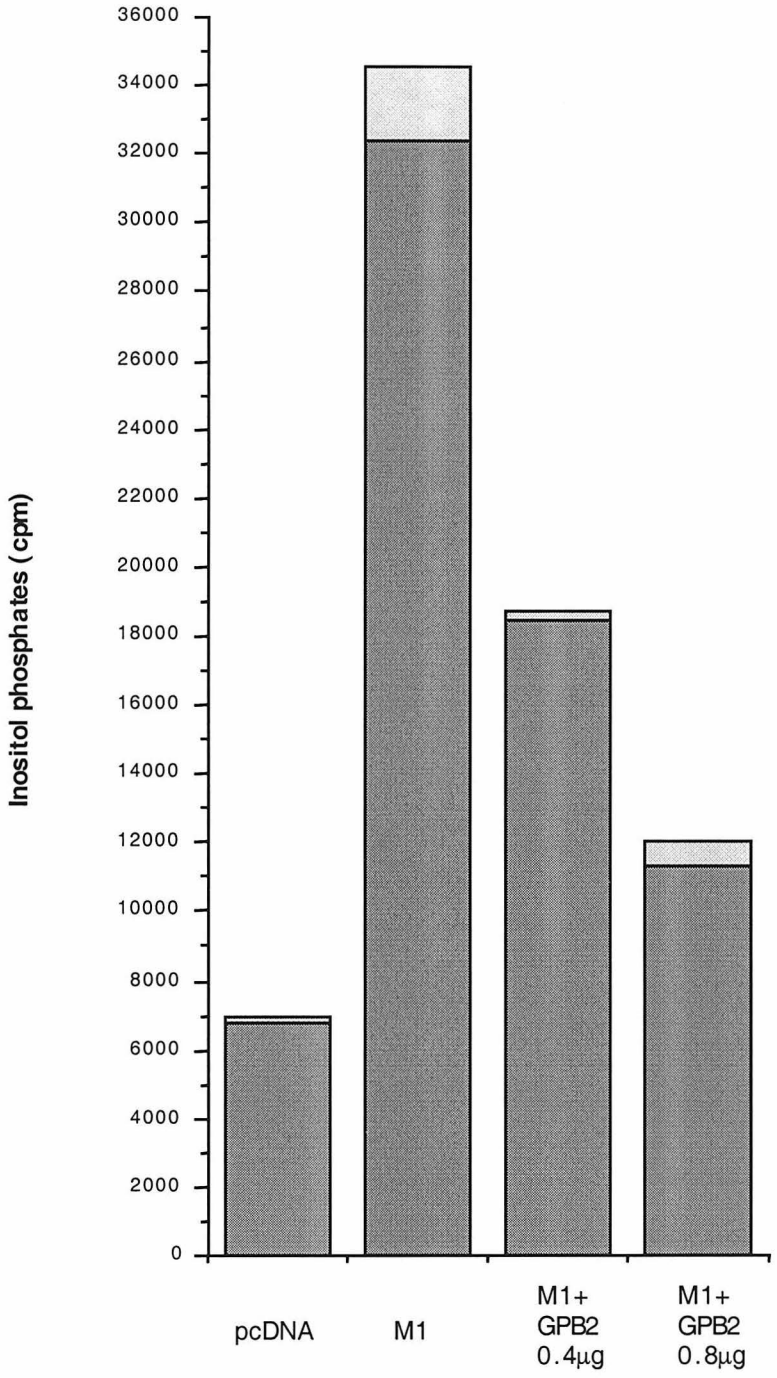

Inositol phosphates (cpm)
36000
34000
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
pcDNA
M1
M1+ GPB2 0.4µg
M1+ GPB2 0.8µg

Figure 4

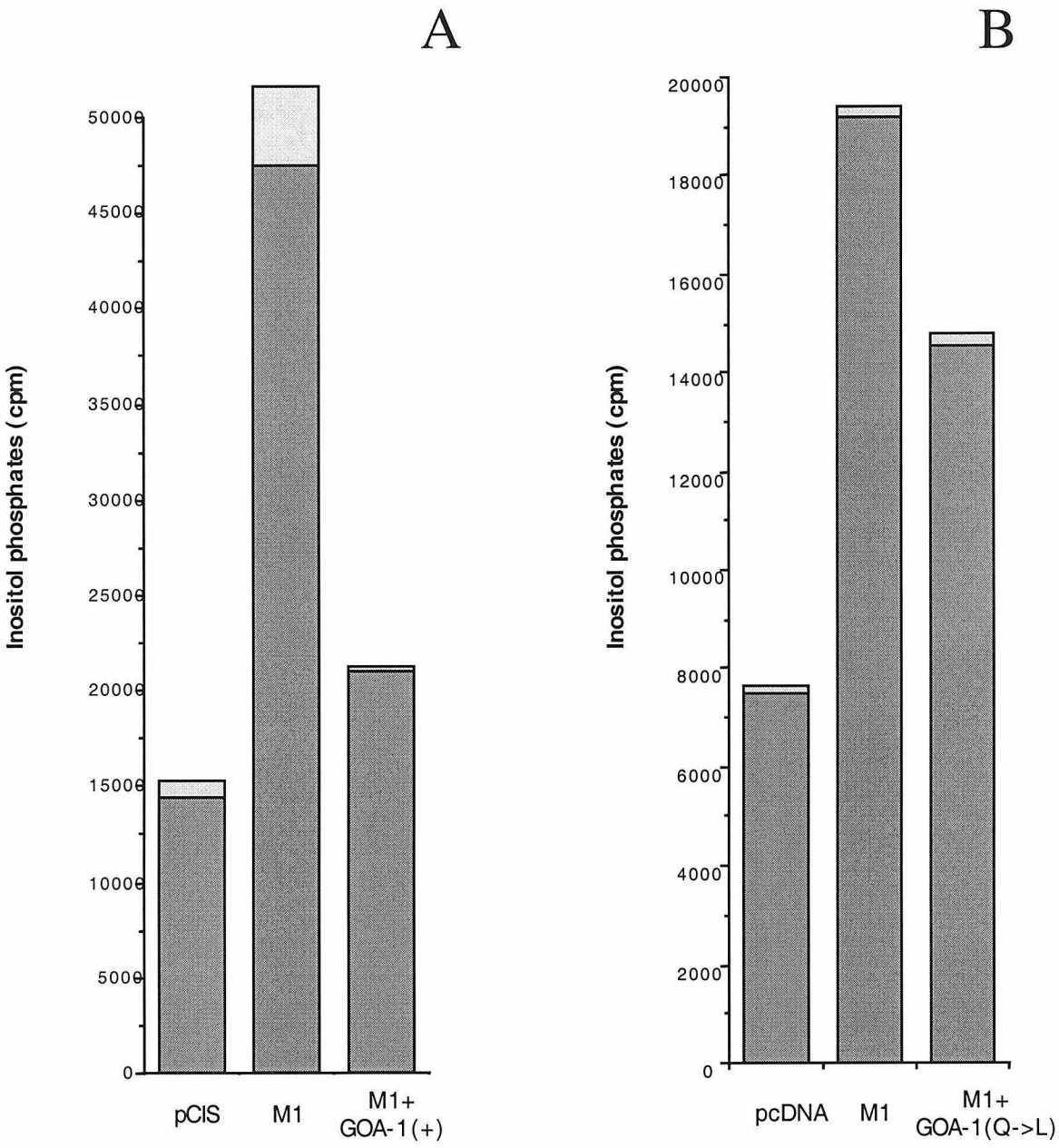
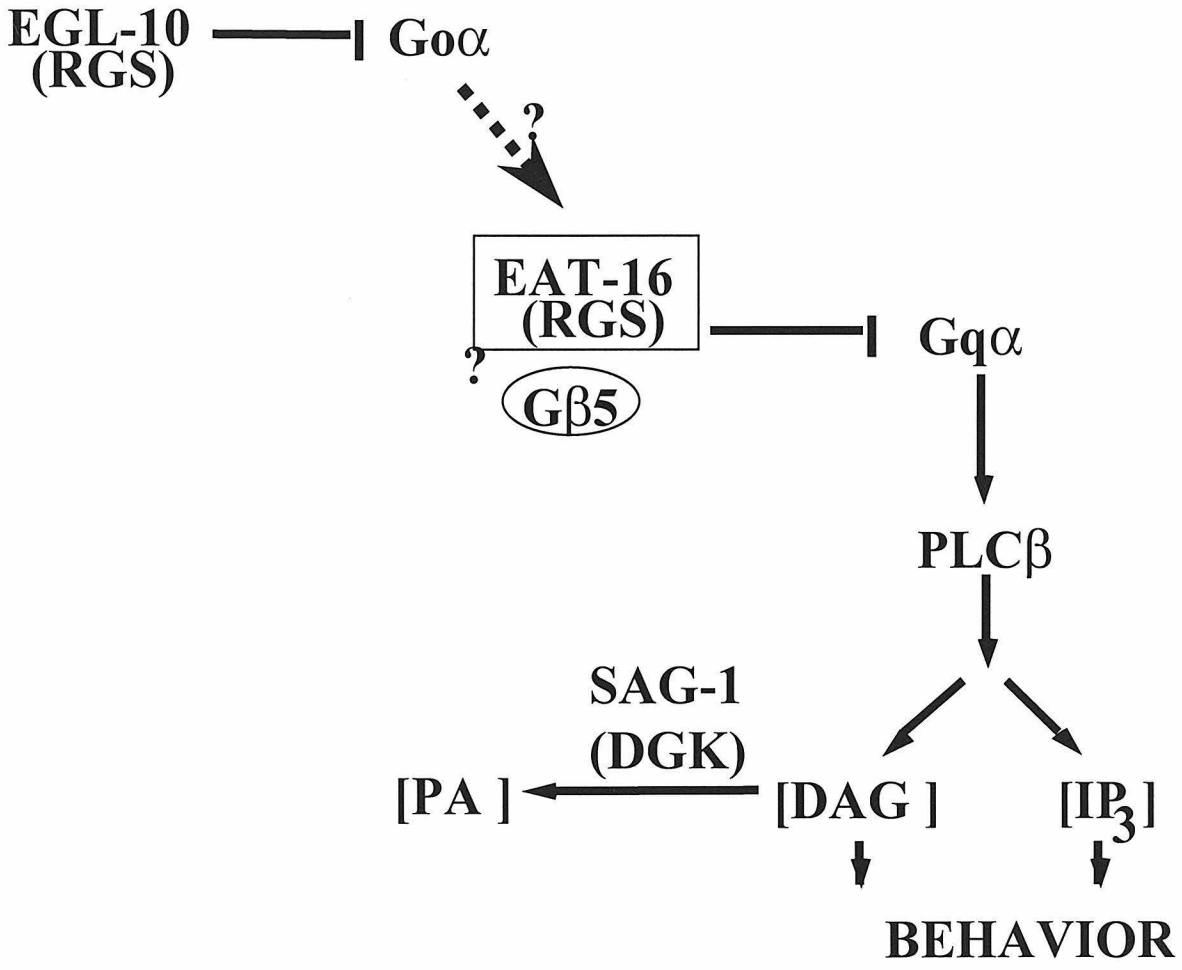

A
B
Inositol phosphates (cpm)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
pCIS
M1
M1+ GOA-1(+)
Inositol phosphates (cpm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
pcDNA
M1
M1+ GOA-1(Q->L)

Figure 5


EGL-10
(RGS)
Goα
?
EAT-16
(RGS)
?
Gβ5
Gqα
PLCβ
SAG-1
(DGK)
[PA]
[DAG]
[IP3]
BEHAVIOR

Summary

Why use a heat shock promoter?

Overexpression of *goa-1* causes *C. elegans* defects in locomotion and egg-laying (Mendel et al., 1995). The strain *syIs9* is the integrated transgenic line overexpressing *goa-1* with Q205->L mutation under the control of Go promoter. The Q->L mutation in Gα has been studied biochemically to constitutively activated Gα. Thus the overexpressed GOA-1 protein should not interact with βγ subunits, unless there is a more complicated mechanism. Although phenotype of *syIs9* is easy to score, there are two technical problems preventing it as the parental strain for mutagenesis and suppressor screen. First, the lethargic and egg-laying defective (Egl-d) phenotype of *syIs9* is progressive. Young animals are very likely to be misidentified as suppressors. Second, possibly due to the toxicity of overexpressed GOA-1, the integrated strain *syIs9* has a high frequency of losing the transgene, which may be mistaken as suppressors.

Thus we used strain *syIs17* to perform mutagenesis. *syIs17* is an integrated transgenic line of Q205->L activated Goα under the control of a heat shock promoter (Mendel et al., 1995). Without heat shock, *syIs17* animals have wild type appearance. Heat shock induces phenotypes more severe than *syIs9*. After heat shock, animals progressively slow down in movement and feeding. Four hours after heat shock the phenotype become obvious because all the *syIs17* animals become paralyzed, stop feeding and stop egg-laying. We performed EMS or UV-psoralen mutagenesis, on non-heat shocked *syIs17* animals, and selected suppressors in F2 progeny by heat shock. Suppressors were scored by the rescue of lethality caused by *syIs17*.

Classify suppressors

For a screen of 21,000 gametes after EMS mutagenesis and another 11,000 gametes after UV-psoralen mutagenesis, 24 independent suppressor alleles were isolated. All the suppressors are recessive. Eight complementation groups were specified and named as *sag-1*, *sag-2*, *sag-3*, *sag-4*, *sag-5*, *sag-6*, *sag-7* and *sag-8*.

It was found later that *sag-1* is independently isolated by another Lab, cloned and named as *dgk-1* (Lackner et al., 1999). The only allele of *sag-2*, *sy438,* failed to complement *eat-16 (ad702)*, a gene originally identified as a feeding mutant by L. Avery in 1993. There is only one allele of *sag-2/eat-16* isolated from the screen. M. Robazack and J. Thomas identified two more alleles (*sa609* and *sa735*) of *eat-16* when looking for suppressors of activated Calmodulin Kinase II (*unc-43*).

Since we designed the screen using heat shock promoter, it is expected that we would get genes either function in G protein signaling or in heat shock response. Thus we performed experiments to test which category does the suppressors belong to. The first is to examine the heat shock induced GOA-1 expression level in parental stain and all the suppressor strains. *sag-1*, *eat-16, sag-6* and *sag-7* do not affect heat shock GOA-1. *sag-1/dgk-1* and *eat-16* (Class I) are similar to *goa-1* mutants in behavior. They are all hyperactive and egg-laying defective. The other two genes in this category seem to function differently from Class I. *sag-6* has wild type appearance. *sag-7* is dumpy, lethargic, and egg-laying defective.

Cyclin L in heat shock induced protein expression

We found four loci significantly reduce the heat shock induced protein expression level. They are *sag-3*, *sag-4*, *sag-5* and *sag-8*. One group (Class II) is *sag-4* and *sag-8*. Defective animals are wild type in appearance. Another group (Class III) is *sag-3* and *sag-5*. They are egg-laying defective. Western analysis indicated that mutations in these genes partially suppress activated Goα by decreasing heat-shock induced protein expression. Although endogenous GOA-1 expression is not affected, heat-shock induction of GOA-1 decreased in the suppressor strains.

We cloned *sag-4* based on its mapping position and found that it encodes *C. elegans* cyclin L, a novel type of cyclin with unknown function. *sag-4* also has high identity to cyclin T, K or C, which was identified as a subunit of TFIIH, part of RNA polymerase II complex and functions in basal transcription (Edwards et al., 1998, Fujinaga et al., 1998,

Rickert et al., 1996),. Transgenes with *hsp16-2* promoter can be suppressed by *sag-4*. However transgenes under other promoters are not affected by *sag-4* mutations.

The sequence similarity, western analysis and genetic results suggest that *sag-4* mutations suppress heat-shock GOA-1 phenotypes by preventing heat- shock mediated transcription in *C. elegans*. We propose that cyclin L is the type of cyclin acting in TFIIH during heat-shock induced mRNA transcription, which carries function similar to cyclin T, K or C during basal transcription. The other suppressor loci identified in the screen, *sag-3*, *sag-5* and *sag-8*, might also be involved in a similar process

RGS protein acting on Gq suppress activated Go

Mutations in two genes, *sag-1* and *eat-16*, were identified as suppressors of activated GOA-1 (Go) in *C. elegans*. Animals that defective in either *sag-1* or *eat-16* display a hyperactive phenotype similar to that of *goa-1* loss-of-function mutants. Double-mutant analysis suggests that both *sag-1* and *eat-16* act downstream of or parallel to G_O and negatively regulate Gq signaling.

sag-1 encodes a diacylglycerol kinase (Lackner et al., 1999), and we found *eat-16* encodes a regulator of G protein signaling (RGS) most similar to the mammalian RGS7 and RGS9 proteins. Although *eat-16* is identified as a suppressor of Go, our genetic and cell culture evidence indicate that *eat-16* functions as a RGS protein for Gq. *eat-16* suppress *egl-30*(Gq) loss-of-function mutants allele-dependently, which is consistent with the direct-binding theory of RGS protein with Gα. *eat-16*(xs) suppress overexpressed *egl-30*(wild type) but not overexpressed *egl-30* with the Q205->L mutation, which is consistent with biochemical evidence that the Q->L mutation in Gα makes the alpha subunit insensitive to RGS. Also, transfecting EAT-16 cDNA to mammalian cells decrease PLC activity induced by mammalian Gq or *C. elegans* EGL-30.

Animals defective in both *sag-1* and *eat-16* are inviable, but reducing Gq function restores their viability, suggesting that the lethality of the *eat-16; sag-1* double mutant is

due to excessive production of second messengers downstream of Gq such as diacylglycerol, and lowering *egl-30* activity decreases DAG level which restores viability. Analysis of these mutations suggests that the Go and Gq pathways function antagonistically in *C. elegans*, and that Go might negatively regulate the Gq pathway, perhaps via EAT-16 or SAG-1.

Antagonism between Go and Gq pathways in *C. elegans*

C. elegans Go and Gq pathways regulate locomotion and egg-laying behavior in a opposite way. Mutant animals with loss-of-function *goa-1* or gain-of-function *egl-30* are hyperactive and egg-laying constitutive. While both *goa-1*(xs) and *egl-30*(lf) cause animals to be lethargic and egg-laying defective. Similarly, negative regulators of these Gα also cause opposite phenotypes. *egl-10* is the RGS protein for *goa-1* (Koelle and Horvitz, 1996) and *egl-10*(lf) animals are egg-laying defective. *eat-16* is the RGS protein for *egl-30* (Hajdu-Cronin et al., 1999) and *eat-16*(lf) animals are hyperactive. Double mutants built with defects in Go and Gq signaling indicate Gq is epistatic to Go at least for most phenotypes.

We speculated that Go and Gq pathway antagonize each other to regulate same behaviors. However it has been difficult to testify the actual relationship between the two pathways. The parallel model is that Go and Gq pathways function in parallel to regulate locomotion and egg lay. According to this model, the epistatic effect is because Gq signal is more dominant than Go. While linear model propose that Go acts upstream of Gq. thus *egl-30* should be epistatic to *goa-1*.

However, it is hard to distinguish these two models. *goa-1*, *egl-30*, *egl-10* and *eat-16* have similar expression patterns (Hajdu-Cronin et al., 1999, Koelle and Horvitz, 1996, Mendel et al., 1995) (C. Bastiani pers. comm.). Although more detailed experiments are required to demonstrate that these genes truly exist in the same cells, it seems very likely the two pathways cross talk or act linearly. The difference between the two models can be justified by elaborate genetic characterization. Or they can be distinguished by

biochemical analysis of intermediate products of the pathways. We reconstituted the system in mammalian cell culture by co-transfecting elements in Go or Gq pathway into COS7 cells. Results indicate that RGS protein *eat-16* negatively regulate PLC activity induced by mammalian Gq/G11 or *C. elegans egl-30*. *C. elegans* Gβ5 homologue GPB-2 interact with EAT-16 in vitro when expressed as recombinant proteins (C. Bastiani and P. Snow, pers. comm.). GPB-2 also negatively regulates PLC activity by Gq/G11, indicating it may participate in the function of EAT-16 or other endogenous RGS proteins. *C. elegans* GOA-1 also negatively regulate PLC activity by Gq/G11. The result indicates the effect of GOA-1 on Gq signaling. It also favors the model that Go acts upstream of Gq pathway.

Closing sentence:

Two major topics are discussed in my thesis: Heat shock response and G protein signaling. We identified cyclin L (*sag-4*), as the candidate for the missing link between HSF and RNA polymerase II during heat shock induced transcriiption. And we cloned RGS7 protein EAT-16, which links together two major G proteins in *C. elegans*, Despite limited study we did, our discovery opened the door to a broader picture of current knowledge towards the area. And it is what we hope, that people who based on our work will solve the mystery. What we contributed, will be one of the tiny steps for knowing the truth.

References:

Edwards, M.C., Wong, C. and Elledge, S.J. (1998). Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity. Mol Cell Biol *18*, 4291-300.

Fujinaga, K., Cujec, T.P., Peng, J., Garriga, J., Price, D.H., Grana, X. and Peterlin, B.M. (1998). The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. J Virol *72*, 7154-9.

Hajdu-Cronin, Y.M., Chen, W.J., Patikoglou, G., Koelle, M.R. and Sternberg, P.W. (1999). Antagonism between G(o)alpha and G(q)alpha in Caenorhabditis elegans: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. Genes Dev *13*, 1780-93.

Koelle, M.R. and Horvitz, H.R. (1996). EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. Cell *84*, 115-25.

Lackner, M.R., Nurrish, S.J. and Kaplan, J.M. (1999). Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. Neuron *24*, 335-46.

Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin, Y.M., Simon, M.I., Plasterk, R.H. and Sternberg, P.W. (1995). Participation of the protein Go in multiple aspects of behavior in C. elegans. Science *267*, 1652-5.

Rickert, P., Seghezzi, W., Shanahan, F., Cho, H. and Lees, E. (1996). Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene *12*, 2631-40.