Negative regulators of the *let-23* EGF receptor in *Caenorhabditis elegans* vulval differentiation

Thesis by

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In partial fulfillment of the requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1993

(Submitted May 10, 1993)

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Acknowledgments:

None of this would be possible (for more reasons than the obvious) without my parents. I'd like to thank them for all that they have done for the past twenty-eight years.

I'd also like to thank Dr. Paul Sternberg, who has been a fantastic advisor. He has shown an amazing mix of patience and enthusiasm and has been invaluble in my education. I regret leaving his lab.

The members of my committee, Dr. Howard Lipshiz, Dr. Ed Lewis, Dr. David Anderson and Dr. Scott Emr have been very helpful as well.

I coouldn't possibly thank all of the people here who have helped me make it through. The members of Paul's lab and my friends throughout the department have been an immeasurable help to me, scientifically and otherwise. Three must be singled out. Thank you Phoebe, Helen and Russell for all of the help, scientific and otherwise. The three of you kept me going for all these years. Thanks to all of you.

Vulval induction in *C. elegans* is an example of the use of an EGF (Epidermal Growth Factor) mediated signal transduction system. At least five genes are involved in the negative regulation of vulval induction.

Mutations at the silent locus *sli-1* (suppressor of lineage defect) are sufficient to suppress all of the phenotypes associated with hypomorphic alleles of *let-23*. *sli-1* functions to modify the activity of *let-23* but muations at *sli-1* do not bypass the requirement for *let-23*. Based on the phenotypes of animals bearing mutations at *sli-1* and other genes, *sli-1* may function at or near the *let-23* or *sem-5* step of vulval differentiation.

Null alleles of the pleiotropic locus *unc-101* cause a number of mutant phenotypes including neural defects and suppression of the vulval defects associated with some weak *let-23* mutations. These *unc-101* mutations interact with mutations in other genes required for proper vulval differentiation but do not act as generalized suppressors. This locus has been cloned and encodes the *C. elegans* homolog of the Golgi-associated clathrin adaptor protein AP47.

Animals mutant for both *unc-101* and *sli-1* display excessive vulval differentiation. Animals mutant at only one of these loci display no vulval abnormalities. This excessive vulval differentiation requires the inductive signal and functional *let-23*, suggesting that *sli-1* and *unc-101* function to negatively regulate the response to the inductive signal, rather than the basal activity of *let-23*.

Rare mutant alleles at *lin-2, lin-7*, and *let-23* result in excessive vulval differentiation. These alleles are genetically similar to more common alleles of these genes which result in the failure to differentiate vulval tissue. These three genes apparently are required for the activation of both positive and

negative regulators of vulval differentiation.

A number of negative regulators function to control the activity of *let-23*. At least three pathaways of negative regulation have been genetically identified. These negative regulators act to limit the response to a growth or differentiation factor.

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Chapter 1

Negative regulation of EGF receptor mediated signal transduction

Signal transduction systems allow cells to communicate with each other and respond to their environment by undergoing changes in their behaviors. Growth factor signaling allow cells to respond to environmental cues by proliferating or differentiating. One classic example of growth factor mediated signal transduction is the Epidermal Growth Factor response. This signal transduction pathway is highly conserved and functions in the development of mammals, *Drosophila,* and nematodes (Figure 1). This system was independently isolated in all three systems. It has been biochemically characterized in mammalian systems, and genetically identified in *Drosophila* and the nematode *C. elegans*. These three systems have all allowed the identification of novel aspects of the pathway, each according to their strengths. Biochemical studies have identified enzymatic activities and targets and proteins that associate physically. Genetic studies have confirmed the order of activity of various genes and identified additional genes acting in the process. These studies have contributed a great deal of knowledge regarding the mechanism of signal transduction through this system. However, very little is known of the down-regulation of this pathway. Biochemical studies have established that sensitivity to ligand decreases as cells are exposed and respond to the ligand. A number of processes are involved in this desensitization. Internalization and degradation of the receptor as well as phosphorylation of the receptor have been implicated in desensitization. The genes involved in this desensitization have proven difficult to identify. Only phospholipase C-γ and its indirect target, protein kinase C, have identified roles. This is far less than the

number of genes which must function in this process. Identification of more genes acting in desensitization of the receptor would further our understanding of this process. A genetic approach may identify gene products required for this process which are not identifiable using biochemical techniques. We have identified and characterized two genes which may function to negatively regulate the Epidermal Growth Factor-receptor in *C. elegans*.

The nematode *Caenorhabditis elegans* is well suited to genetic analysis. It is small, free living, and has a very short generation time, allowing the manipulation of large numbers of animals in a small space and a short period of time. Hermaphrodites are self-fertile, and males are relatively easy to recover and are able to mate with a high efficiency, allowing easy manipulation and maintenance of mutant alleles. Most of the tissues of the worm are dispensible, as ablation of large numbers of cells or mutations that disrupt the formation or function of several tissues do not result in inviability. For instance, mutations that disrupt muscular structure, vulval development, or neural develpment or function do not result in inviability. The cell lineage of the hermaphrodite is essentially invariant. Some of this is probably "hard-wired," in that most lineages are not easily disruptable by cell ablation or any of the characterized mutations. However, it is becoming increasingly apparent that a great deal of the invariance is due to highly reproducible cell-cell interactions and redundancy of signalling cells. The study of cell-cell interactions is therefore quite approachable in this system. Interactions that instruct specific cells to assume the proper fates may be studied by a combination of ablation (using a laser microbeam) and genetic experiments combined with lineage analysis. Molecular analysis of the loci

identified in these genetic screens is simplified greatly by the state of the analysis of the *C. elegans* genome. Most of the genome is present in ordered overlapping cosmid and YAC clones (contigs) (Coulson et al., 1988, Coulson et al., 1986) and eventually the entire genome will be sequenced (Sulston et al., 1992). Transgenic technology allows the introduction of cloned or mutated genes into animals of various genetic backgrounds. The combination of these powerful genetic, developmental, and molecular tools allows the rapid identification and characterization of novel loci acting in identified pathways.

C. elegans

C. elegans vulval differentiation

The hermaphrodite vulva is an example of a tissue that is formed as the result of cell-cell interactions. The anchor cell of the somatic gonad is necessary and sufficient for the induction of this process (Kimble, 1981) (Figure 2). This cell produces an inductive signal which is received and responded to by the P(3-8).p epidermoblasts (these six cells are referred to as Vulval Precursor Cells, or VPCs). The three cells most proximal to the anchor cell respond to the inductive signal and differentiate into vulval tissue, while the other three form undifferentiated hypodermis (Figure 2). The extent of vulval differentiation and fate assignment of these cells is refined by lateral signalling (Sternberg, 1988). The eventual outcome of these two signalling systems is the generation of a nearly invariant pattern of cell fates. P3.p, P4.p, and P8.p (these names describe the ancestry of the cell in question, for instance, P3.p is the posterior daughter of the cell P3, the period denotes that the division of P3 to P3.a and P3.p was post-embryonic)

divide once longitudinally (with respect to the anterior/posterior body axis) and join the hypodermal syncytium. This fate is considered to be non-vulval and is the ground state in the absence of inductive information from the anchor cell. P5.p and P7.p each undergo two longitudinal divisions followed by a third round of division in which P5.paa, P5.pap, P7.ppa and P7.ppp divide longitudinally, P5.ppa and P7.pap divide transversely, and P5.ppp and P7.paa do not divide, giving rise to seven grand-daughters each. All fourteen (seven from P5.p, seven from P7.p) of these progeny differentiate as vulval tissue. P6.p also undergoes three rounds of division (the last round consists of four transverse divisions) generating eight vulval nuclei (Figure 2) (Sulston and Horvitz, 1977). Each of these developmental programs is referred to as a sublineage (Chalfie et al., 1981, Sternberg, 1982, Sulston et al., 1983). This term describes a pattern of divisions (frequently repeated by a number of similar cells) that is considered to reflect a determined state of the progenitor of these cells. Sublineage division patterns are not disrupted by ablation of a subset of the daughters (W. Katz and P. Sternberg, pers. comm.), suggesting that the division pattern may be "hard-wired" and not reliant on ongoing cell-cell interactions. These sublineages may be used as a basis for the generation of similar but non-identical groups of cells. For instance, the P(1-12).a neuroblasts all undergo a similar sublineage. The number and type of progeny cells is subsequently refined by cell death, leading to the generation of several different fates using only a single sublineage (Sulston, 1976).

Extensive studies have been carried out on the assignment of fate in this group of cells. If P6.p is ablated at a sufficiently early stage, a near neighbor (P5.p or P7.p) will undergo the lineage that is normally executed by P6.p. Similarily, if P5.p (or P7.p) is removed, a neighboring epidermal cell (P4.p or

P8.p) executes the lineage normally associated with the missing cell (Figure 2) (Sulston and White, 1980). Using such strategies, the sublineages of these cells have been assigned as primary, secondary, and tertiary fates. The primary fate (normally executed by P6.p) is so named because it is the fate which will be executed at the expense of all other fates. Likewise, the secondary fate (normally executed by P5.p and P7.p) will be executed preferentially to the tertiary (uninduced) fate. P3.p, P4.p, and P8.p are considered members of this equivalence group because these cells are capable forming vulval tissue if other VPCs are ablated (or in certain mutant backgrounds, see later). P2.p and P9.p generally do not form vulval tissue or undergo the tertiary fate. These cells do not divide and fuse to the hypodermal syncytium.

As mentioned earlier, ablation of cells at a later stage (after the first round of division) does not disrupt the lineage executed by neighboring cells, suggesting that cell-cell interactions play a very minor role at this point or that the cell debris is sufficient to fulfill the signalling function normally required form the ablated cell. This is interpreted to imply that cell fate in the vulva is determined at the one cell stage (i.e., the Pn.p cells, rather than the Pn.px cells). Consistent with this, the VPCs in animals bearing mutations giving rise to abnormally long bodies or dorsally displaced gonads (both of which disrupt the relative positioning of the anchor cell and the VPCs) do not display "split" lineages. The same sublineages are executed (either 1°, 2°, or 3°, rather than 1°/2° hybrids) (Sternberg and Horvitz, 1986, Thomas et al., 1990). This result would be expected if the Pn.px cells have inherited a fate assignment from their precursors that cannot be disrupted by altering the spatial relationships between the anchor cells and the Pn.px

cells. However, it is possible that the Pn.px cells are the cells which undergo determination and that the siblings cooperate in fate assignment (i.e., if P6.pa assumes half of what we consider a primary fate, then it forces its sister to undergo the other half). Preliminary evidence suggests that ablation of a Pn.px cell does not affect the lineage of its sister (W. Katz and P. Sternberg, pers. comm.). If this interaction occurs soon after the sister cells are born, ablation is unlikely to reveal the interaction. Although the possibility that the Pn.px cells are responsive to cell-cell interactions cannot be ruled out, the simplest model is that fate determination occurs at the Pn.p rather than the Pn.px stage.

A variety of mutations that disrupt proper vulval development have been identified. Mutations that likely represent defects in generation of VPCs, production of inductive information, inhibition of spontaneous differentiation, response and interpretation of the inductive signal, execution of lateral signalling and execution of specific sublineages have been recovered in screens for mutations disrupting the function or appearance of the vulva (Ferguson and Horvitz, 1985, Horvitz and Sulston, 1980). Genetic and anatomical analyses allowed the identification of mutations which may disrupt the inductive process and are required for the formation of either vulval or epidermal tissue. Mutant animals which fail to lay eggs yet have a normal vulva or appear to have no VPCs are assumed to bear mutations affecting other processes in the generation of a functional vulva.

Animals bearing mutations at a number of these loci result in all of the VPCs forming epidermal tissue (the vulvaless, or Vul phenotype). In these mutant animals, eggs are fertilized internally by the hermaphrodite's sperm. These eggs subsequently hatch and devour the parent. This phenotype is

called Vulvaless (Vul), and is assumed to be the result of a mutation in a gene required for production of or response to the inductive signal, provided that the mutation is hypomorphic or amorphic. Animals bearing other mutations are unable to form epidermal tissue or overproduce of vulval tissue. The excess vulval tissue forms pseudovulvae on the ventral surface of the mutant animal, visible as small ventral protrusions on the adult. Since connection of the vulval tissue to the anchor cell is required for the formation of a functional vulva only the tissue near the anchor cell forms a normal vulva. The extra tissue everts to form a psuedovulva after the animal undergoes the molt from L4 to adulthood. This phenotype is referred to as Multivulva, or Muv. These mutations render the formation of vulval tissue independent of the inductive signal (Ferguson et al., 1987) , suggesting that these mutations act in the responsive tissue, rather than acting as negative regulators of inductive signal production in the anchor cell. Using genetic and molecular analyses, these mutations have been ordered into a signal transduction pathway (Ferguson et al., 1987, Han et al., 1990, Han et al., 1993). For the genes which have been cloned, this genetic order is consistent with molecular models based on experiments in other systems (Figure 1).

lin-3, the apparent inductive signal, is similar to Epidermal Groeth Factor

Molecular analysis of several of these loci has proven that this inductive pathway is mediated by a very well studied group of genes. Several of the genes are *C. elegans* homologs of known mammalian proto-oncogenes and the inductive pathway appears to be very similar to EGF mediated pathways studied in other systems. The apparent inductive signal is encoded by *lin-3*

(Hill and Sternberg, 1992). The predicted protein product of this locus includes an extracellular domain that is similar to members of the Epidermal Growth Factor family (Carpenter and Cohen, 1990, Hill and Sternberg, 1992). Animals bearing *lin-3::lacZ* transgenes display β-galactosidase activity in the L2 anchor cell but not in the VPCs (Hill and Sternberg, 1992). Animals bearing *lin-3(+)* transgenes display gonad dependent Muv phenotypes (Hill and Sternberg, 1992), consistent with the overexpression of the inductive signal. Reduction-of-function mutations at *lin-3* result in a decrease in the number of cells responding to the inductive signal (R. Hill and P. Sternberg, pers comm., Sternberg and Horvitz, 1989, Sulston and Horvitz, 1981). However, the loss of responsive cells is graded. If any cell is induced, it is P6.p, which will undergo a 1° or 1° like fate (not a 2° fate) (R. Hill and P. Sternberg, pers. comm.). Thus the inductive information in these animals is still present and graded, but is weaker in its ability to induce differentiation in cells distant from the anchor cell. Mutations in other genes required for vulval development do not result in such a graded pattern. Rather, these mutations frequently result in properly positioned 2° lineages without apparent 1° lineages, (G. Jongeward and P. Sternberg, unpub. obs.) suggesting that the response to the inductive signal is impaired, rather than the production or release of the inductive signal. *lin-3* mutations or overexpression cause defective development of a number of other tissues as well. Mutations at this locus confer L1 lethality, sterility, and male tail defects as well as the described vulval defects (R. Hill, H. Chamberlin, and P. Sternberg, pers. comm.). The development of the P12 neuroectoblast is disrupted in animals bearing transgenes which overexpress the *lin-3* gene product (R. Hill and P. Sternberg, pers. comm.). Thus, *lin-3* encodes a

molecule similar to a growth factor that genetically behaves as one would expect the inductive signal to behave. Mutations at *lin-3* are hypostatic to all other mutations that have been tested (Ferguson et al., 1987, Hill and Sternberg, 1992), which is consistent with *lin-3* acting as the inductive signal.

let-23, the putative receptor for the inductive signal, encodes a homolog of the Epidermal Growth Factor Receptor and controls both positive and negative functions

let-23 encodes a candidate receptor for the *lin-3* encoded inductive signal. *let-23* encodes a putative receptor tyrosine kinase of the Epidermal Growth Factor receptor subfamily (Aroian et al., 1990, Schlessinger and Ullrich, 1992, Figure 3). *let-23* mutations confer the same set of mutant phenotypes as *lin-3* mutations(Aroian and Sternberg, 1991). *let-23* reduction-of-function mutations disrupt the proper determination of the P12 neuroectoblast (Aroian and Sternberg, 1991). A viable *let-23* mutation is epistatic to a *lin-3(+)* transgene (Hill and Sternberg, 1992), suggesting that *lin-3* functions as an activator of *let-23*. Most *let-23* mutations appear to be simple reduction or loss-of-function alleles (Aroian and Sternberg, 1991). The phenotypes associated with these alleles are apparently the result of a failure of signal transduction via this molecule, rather than signal independent activation of this gene product in either the normal or ectopic cells. Mutations in the kinase domain or the ligand binding domain of the predicted protein result in severe defects (generally L1 lethality as well as strong vulval defects in *trans* to other mutations at the locus) (Aroian et al., 1993), suggesting that both of these regions are essential for proper function of the *let-23* gene product. Mutations that truncate the C-terminal region of this gene result in less

severe effects on development of most tissues (Aroian et al., 1993). Some of these weak mutations have no apparent affect on some of the tissues which are defective in animals bearing stronger *let-23* genotypes (Aroian and Sternberg, 1991). These data suggest that the C-terminus is dispensible, as removal of this region does not prevent the functioning of the receptor. One allele, *let-23(n1045)*, a mutation in the C-terminus (Aroian et al., 1993), displays a novel phenotype at high temperatures. Animals homozyogus for this allele display excessive vulval differentiation at 25° (Aroian and Sternberg, 1991 and Chapter 5). At 15°, this allele confers phenotypes similar to those seen in animals bearing weak reduction-of-function alleles (Aroian and Sternberg, 1991 and Chapter 5). All vulval differentiation in *let-23(n1045)* animals is dependent on the presence of inductive information (Aroian and Sternberg, 1991 and Chapter 5), suggesting that this allele does not produce a protein that is ligand independent, rather one that is hypersensitive or hyperresponsive to the inductive signal. This may be due to a failure of this gene product to activate negative regulators. A heteroallelic combination involving two other *let-23* alleles also results in this phenotype (Aroian and Sternberg, 1991), suggesting that this phenotype is not a novel phenotype associated with a single allele. Thus, one of the roles of the *let-23* gene product is probably the activation of negative regulators. Mosaic analysis suggests that *let-23* functions in the VPCs and not in the anchor cell (M. Koga and Y. Ohshima, pers. comm.). Animals bearing transgenes containing the *let-23* gene display no obvious phenotype (aside from rescue of a chromosomal *let-23* mutation, Aroian et al., 1990), suggesting that either the concentration of this gene product is not rate-limiting for vulval induction or that the expression of this gene is autoregulated. This is surprising for two

reasons. One might expect that the overexpression of *let-23* might disrupt vulval differentiation. If *lin-3* is limiting and is required to induce the presumptive 2° cells, then one might expect that overexpression of *let-23* would cause most of the *lin-3* signal to be bound by the presumptive 1° cell, resulting in a reduction in the extent of induction. It could also be possible that overexpression of *let-23* would result in excessive vulval differentiation, as (1) the *lin-15* inhibitory signal (see later) might be overwhelmed by overexpression of *let-23,* resulting in an increase in the inductive signal-independent differentiation of vulval tissue, or (2), the overexpression of *let-23* might overcome other (presumably intracellular) negative regulators, again resulting in excessive vulval differentiation. The simplest interpretation of these data is that *let-23* is under tight control by the combination of negative regulation via the *lin-15* gene products and other intracellular negative regulators (see the descriptions of *sli-1* and *unc-101* in chapters 2, 3,and 4).

sem-5 encodes a GRB2 homolog that acts after *let-23* and before *let-60 ras*

sem-5 encodes an SH3-SH2-SH3 protein (Clark et al., 1992) similar to that encoded by GRB2 (Lowenstein et al., 1992) and *drk* (Olivier et al., 1993, Simon et al., 1993). SH2 and SH3 domains are believed to be involved in protein/protein interactions, reviewed in Koch et al., 1991. Mutations at the *sem-5* locus confer a number of phenotypes, including sex muscle migration defects, lethality, and failure of vulval tissue differentiation (Clark et al., 1992). This gene is believed to act after *let-23* but before *let-60*, based on a combination of epistasis and molecular experiments. *sem-5* mutations were

recovered as suppressors of *lin-15* and are suppressed by *let-60 ras gf* mutations (Clark et al., 1992), suggesting that this protein acts after *lin-15* but before *let-60 ras*. The ordering of this gene with respect to *let-23* is based entirely on the interaction of GRB2 and the EGF receptor, as no characterized gain-of-function mutations exist for either *let-23* or *sem-5*. This class of protein is believed to act as adaptors between receptor tyrosine kinases and their substrates, probably guanine nucleotide exchange factors (Olivier et al., 1993, Simon et al., 1993), thus acting to regulate the activity of *ras*. *sem-5* is believed to directly interact with *let-23* via binding of *let-23* phosphotyrosines to the *sem-5* SH2 domain. This is based on the interaction of GRB2 with phosphotyrosine domains in the EGF receptor (Lowenstein et al., 1992). If this is the role of *sem-5* in vulval differentiation, then it may be interacting with other genes in at least one other tissue, as neither *let-23* nor *let-60 ras* mutations display a sex-muscle migration defect.

***let-60 ras* acts as a switch**

let-60 encodes a *C. elegans ras* homolog (Bourne et al., 1991, Han and Sternberg, 1990). Several classes of mutations at this locus have been analyzed (Beitel et al., 1990, Han et al., 1990). Loss-of-function mutations at the locus are lethal, masking defects in vulval differentiation and male tail defects (Han et al., 1990). Reduction-of-function mutations confer similar phenotypes, although not all animals bearing such alleles display all of the phenotypes (Beitel et al., 1990, Han et al., 1990). Dominant negative mutations at the locus result in dominant defects in vulval differentiation and the male tail (Han et al., 1990). These mutations are recessive lethal and fail to complement the lethality of the loss-of-function alleles (Han et al.,

1990). These dominant negative mutations were recovered as suppressors of *lin-15*, suggesting that *let-60 ras* acts after *lin-15* (Han et al., 1990). Gain-of-function alleles result in dominant transformations of the epidermal lineages to vulval lineages (Muv). These mutations are capable of suppressing strong mutations at some of the upstream genes such as *let-23* (Han et al., 1990), further supporting the interpretation that this gene acts after *let-23* and *lin-15*. Thus, *let-60 ras* acts as a switch gene acting after *let-23* in vulval differentiation.

lin-45, a raf homolog, acts after *let-60 ras*

lin-45 encodes a *C. elegans* homolog of the *raf* serine threonine kinase (Han et al., 1993, Heidecker et al., 1992). Mutations of this gene have also been recovered as suppressors of *lin-15* (Han et al., 1993). A putative reduction-of-function mutation at this locus confers a Vul phenotype as well as male tail defects and subviability (Han et al., 1993). The subviability of one of these mutations is enhanced by a deficiency of the locus (A. Golden and P. Sternberg, pers. comm.), suggesting that this allele may be a reduction-of-function allele. This mutation partially suppresses the Muv phenotype of a *let-60(gf)* allele (Han et al., 1993), prompting the interpretation that *lin-45 raf* acts downstream of *let-60 ras*. Since this allele is not a null allele and the phenotype of the double mutant is intermediate, this ordering must be viewed as tentative. Animals bearing transgenes containing putative dominant negative mutations of *lin-45 raf* also display defects in vulval differentiation (A. Golden and P. Sternberg, pers. comm.). Further genetic characterization of this locus will hopefully clarify its interaction with other genes in the pathway, including the null phenotype of the locus and the phenotype of an

animal bearing a null allele at this locus and a *let-60 gf* allele.

lin-1 acts after *let-60* and *lin-45* and may encode a transcription factor

lin-1 is the most downstream genetically defined locus in the inductive pathway identified to date. Mutations at this locus result in the constitutive formation of vulval tissue and suppress the vulval defects associated with mutations at *lin-3, let-23, sem-5, let-60,* or *lin-45*(Clark et al., 1992, Ferguson et al., 1987, Han et al., 1990, Han et al., 1993). This gene has been cloned and it encodes a putative DNA binding protein of the Ets family (G. Beitel and R. Horvitz, pers. comm., reviewed in Macleod et al., 1992). Presumably, this gene functions to positively regulate expression of epidermal genes or to inhibit the expression of vulval genes. Further analysis of this locus may suggest the site of action of the *lin-1* gene.

Several other genes regulate this process

lin-15 is a complex locus that apparently acts as a negative regulator of vulval differentiation. This locus comprises two separable, redundant genetic functions ("A" and "B") (Ferguson and Horvitz, 1989) which correlate to two dissimilar transcripts from the locus (L. Huang and P. Sternberg, pers. comm.). Several other loci encode either A or B functions (Ferguson and Horvitz, 1989). *lin-15* is the only locus known to include both A and B functions (Ferguson and Horvitz, 1989). Animals homozygous for both an A mutation and a B mutation display a Muv phenotype, while animals homozygous for only one of these mutations are phenotypically wild-type (Ferguson and Horvitz, 1989). Mosaic analysis of this locus suggests that *lin-*

15 may be partially non-autonomous (Herman and Hedgecock, 1990). The surrounding hypodermal syncytium has been proposed as the site of this *lin-15* activity, as most of the other surrounding tissue (the Pn.a derived neurons, the VPCs themselves, and the gonad) was characterized (Herman and Hedgecock, 1990). It is not clear whether *lin-15* acts at all in the VPCs or the anchor cell, nor is it known whether the non-autonomy observed reflects non-autonomy of one function (A or B) or both functions. Ablation of the gonad does not suppress a *lin-15* mutation, whereas *lin-15* mutations are suppressed by mutations at *let-23*(Ferguson et al., 1987) suggesting that *lin-15* acts to prevent *let-23* activation in the absence of inductive signal. This epistasis is consistent with the non-autonomy of this gene product. The further analysis of this pathway and the identification of the presumptive secreted negative regulator will be helpful in extending the understanding of the control of responsiveness by growth factor antagonists.

Mutations at the *lin-2, lin-7,* and *lin-10* loci are frequently recovered in screens for mutations conferring abnormalities in vulval differentiation (Ferguson and Horvitz, 1985, Horvitz and Sulston, 1980). In contrast to mutations at other genes involved in this process, these mutations do not result in inviability (Ferguson and Horvitz, 1985). Apparent null mutations at these loci also do not completely disrupt the formation of vulval tissue, as animals homozyogus for such an allele frequently display residual vulval differentiation (Ferguson and Horvitz, 1985, Kim and Horvitz, 1990) and Chapter 5. Triply mutant animals also display residual vulval differentiation (E. Ferguson and R. Horvitz, pers. comm.), suggesting that this bypass pathway is independent of any of these genes. The amount of vulval differentiation in these triple mutant animals is only slightly more severe

than that of any of the single mutants (E. Ferguson and R. Horvitz, pers. comm.), suggesting that these three genes act in the same pathway and that the loss of one is functionally similar to the loss of all three. To date, only one of these genes has been cloned. *lin-10* encodes a novel protein with a broad expression pattern (Kim and Horvitz, 1990). Some mutations at two of these loci (*lin-2* and *lin-7*) result in excessive gonad dependent vulval differentiation (the Hyperinduced or Hin phenotype). Four criteria suggest that these alleles are reduction-of-function alleles. First, the Hin phenotype associated with these alleles requires the presence of inductive information. A gain-of-function allele of a gene acting in the VPCs should be anchor cell independent (like the *let-60 gf* alleles, see previous section). Second, these mutations are recessive and fail to complement alleles which cause a Vul phenotype. The *trans*-heterozygote is Vul, suggesting again that these alleles are not hypermorphs. Third, an amber suppressor mutation can suppress an amber suppressible (apparent null) allele of *lin-7* to an excessive differentiation phenotype, suggesting that other mutations at these loci are also able to cause these phenotypes but that this defect is masked by a failure in vulval differentiation. Fourth, animals homozygous for two of these alleles display a Vul, rather than a Hin or wild-type phenotype, suggesting that the cumulative vulval differentiation defects of two of these alleles are sufficiently severe to mask the failure to negatively regulate (see chapter 5).

The process of vulval differentiation acts through a signal transduction pathway including homologs of epidermal growth factor, epidermal growth factor receptor, ras, raf and Ets, as well as novel genes that act as both negative (*lin-15*) and positive (*lin-10*) regulators of the process. The powerful genetics of this system allows the recovery of rare recessive mutations in

large screens to identify more genes in this pathway. My screens have identified mutations at two of the loci described here, an activated *let-60 ras* allele and a *let-23* intragenic revertant. In addition, I have recovered mutations at several unlinked loci which had previously not been identified as acting in the vulval differentiation pathway.

One of the novel genes, *sli-1* (suppressor of lineage defect), is a silent suppressor of *let-23* mutant phenotypes. Null mutations at this locus confer no obvious phenotype, aside from suppression of vulval defects. This locus dispalys dosage sensitivity. In some genetic backgrounds. this locus is haplo-insufficient. Overexpression phenotypes are also observed. *sli-1* mutations are not sufficient to bypass the requirement for *let-23*, suggesting that *sli-1* functions to control the activity of *let-23*, but does not relieve the requirement for functional *let-23*. Mutations at *sli-1* suppress *let-23, sem-5, lin-2, lin-7* and *lin-10,* but do not suppress mutations at *lin-3, let-60* or *lin-45*. One interpretation of these data is that *sli-1* acts at the *let-23* step of vulval differentiation to negatively regulate the activity of *let-23*, either directly or indirectly (see chapter 2).

Two alleles of *unc-101* were also isolated. This locus is pleiotropic, and was previously identified on the basis of other phenotypes associated with mutations at the locus. Apparent null alleles of the gene result in reduced viability, failure in neural outgrowth and function, poor movement, and suppression of mutations in genes required for vulval differentiation. *unc-101* mutations suppress a subset of mutant alleles at *let-23*. Null alleles are not suppressed, and some weak alleles are suppressed, while other weak alleles are enhanced or only slightly suppressed. Some of the other phenotypes associated with *let-23* mutations are suppressed (sterility), while

other phenotypes are not suppressed (lethality). This locus has been cloned and encodes a homolog of the *trans*-Golgi associated clathrin adaptor protein AP47, which is believed to act in the sorting of proteins to the endosome (see chapter 3).

Mutations at neither *sli-1* nor *unc-101* confer a defect in vulval differentiation in an otherwise wild-type background. However, animals homozygous for mutations in both *unc-101* and *sli-1* display a novel phenotype. These animals display excessive vulval differentiation. Vulval differentiation in these animals is partially gonad dependent and partially gonad independent. The residual vulval differentiation in these gonad ablated animals is dependent on the presence of functional *let-23*, and appears to reflect the presence of inductive signal "leaking" from another tissue, suggesting that the function of *unc-101* and *sli-1* is primarily to down-regulate the response to the inductive signal, rather than to down-regulate the basal activity of *let-23* (see chapter 4).

C. elegans is a good system for identifying regulators of this pathway. Vulval differentiation is a very sensitive bioassay, in that it is easy to screen for slight alterations in the sensitivity to the inductive signal. A number of mutations in these genes are highly penetrant and expressive, allowing one to screen large numbers of animals for modifiers. Recessive mutations are easier to isolate in this system than in *Drosophila melanogaster*. As yet, there are not a great many gain-of-function mutations affecting this process. Such mutations would allow the analysis of the epistasis of genes such as *lin-2, lin-7, lin-10* and *let-23*. These genes are not convincingly ordered with respect to each other and gain-of-function mutations at these loci would clarify their interactions.

Drosophila

sevenless/DER and inductive signaling in *Drosophila*

Several receptor tyrosine kinases have been identified genetically on the basis of their roles in *Drosophila melanogaster* development (reviewed in Shilo, 1992). Two of these are DER, the *Drosophila* EGF receptor, and *sevenless*. The *D. melanogaster* homolog of the EGF receptor was identified genetically on the basis of three different phenotypes. Gain-of-function alleles (*Ellipse*) disrupt proper pattern formation in the eye (Baker and Rubin, 1989). Reduction-of-function alleles (*torpedo*) were recovered on the basis of a ventralized phenotype (Price et al., 1989), and apparent null alleles (*faint little ball*) were recovered as embryonic lethals (Schejter and Shilo, 1989). *sevenless* mutant animals have no R7 photoreceptor cells but display no other phenotype (Harris et al., 1976). This gene encodes a receptor tyrosine kinase which interacts with *bride of sevenless*, its apparent ligand (Hart et al., 1990, Reinke and Zipursky, 1988).

As *sevenless* is dispensable for viability and affects the eye, very powerful genetic screens can be performed to identify interacting genes. In one series of screens, animals bearing a temperature sensitive *sevenless* allele (Simon et al., 1991)were raised at a temperature just high enough to allow the development of R7 cells, thus sensitizing the development of these cells to the dosage of other genes involved in this signal transduction pathway. From the progeny of mutagenized animals, phenotypically rough-eyed mutants were selected and analyzed. Similarly, a screen for enhancers was performed at a temperature slightly below that required for the development of R7 cells,

allowing the recovery of mutations in negative regulators and antagonists of this developmental pathway as dominant suppressors (conferring a more wild-type phenotype) of the *sevenless* mutant phenotype (Gaul et al., 1992). Mutations at some of these loci have been recovered in screens for rough-eyed mutants in wild-type backgrounds as well. Both screens identify genes involved in the *sevenless* signal transduction pathway. The identified mutations were subsequently characterized for their interactions with the *Ellipse* allele of DER (Simon et al., 1991). Mutations which interact with both *sevenless* and *Ellipse* are assumed to define loci which are common to these two receptor tyrosine kinase signal transduction pathways. I will concentrate on these loci. Recently, several of the loci shown to be involved in signaling from these receptors have also been shown to be involved in signaling through *torso*, a third receptor tyrosine kinase required for development (Doyle and Bishop, 1993, Lu et al., 1993). It seems increasingly likely that many of the components involved in any one of these pathways will be involved in a number of pathways. The genes identified in the *sevenless* pathway which also act in the DER pathway include *drk*, (a *sem-5* homolog) (Olivier et al., 1993, Simon et al., 1993), the regulators of *ras* activity guanine nucleotide exchange factor (GNEF) (Bonfini et al., 1992) and GTPase activating protein (GAP) (Gaul et al., 1992), *ras* (Fortini et al., 1992, Simon et al., 1991), and *raf* (Dickson et al., 1992). Unfortunately, the interactions of many of these genes has yet to be presented. The epistasis of these mutations and *sevenless* and *Ellipse* is clear, but much of the subsequent epistasis is based on molecular identity.

The *Drosophila* EGF receptor is required for proper development

of many tissues and may interact with *spitz*

DER is involved in a number of developmental processes in the fly. In general, mutations at this locus are not tissue specific, but several are tissue-preferential (Clifford and Schupbach, 1989). As is seen with *let-23,* DER activity is required for viability, as well as for the proper development of post-embryonic structures. *spitz* is a candidate ligand for DER. *spitz* encodes a member of the Epidermal Growth Factor family (Rutledge et al., 1992) and may activate DER in *vitro.* Some of the phenotypes associated with mutations at DER are similar to those conferred by mutations at *spitz.* One possible explanation for this observation is that DER has two or more ligands. This is consistent with the role the EGF receptor is thought to play in mammalian development. The mammalian EGF receptor recognizes both EGF and Transforming Growth Factor–α (TGF-α) in *vitro,* and this interaction is probably relevant in *vivo.* Gene disruption studies of the EGF receptor and TGF-α also display dissimilar phenotypes. Disruption of the EGF receptor results in lethality at the blastocyst stage of development (T, Magnuson, pers. comm.), while disruption of TGF-α is viable and confers only a few defects (Luetteke et al., 1993, Mann et al., 1993). Presumably, either EGF activates EGF receptor in most other tissues or both TGF-α and EGF activate the receptor in most tissues. The phenotypes associated with the various mutant alleles of DER and *spitz* and the molecular identities of these genes suggest that they might interact as receptor and ligand.

GAP, a negative regulator of *ras* activity

One of the loci that can be mutated to suppress a *sevenless* mutation is the *Gap1* locus (Buckles et al., 1992, Gaul et al., 1992, Rogge et al., 1992). This

gene encodes a *Drosophila* homolog of the Ras GTPase activating protein (Gaul et al., 1992), which is believed to function in the control of *ras* activity by catalyzing the GTPase activity of *ras*, resulting in the conversion of *ras*-GTP (active) to *ras*-GDP(inactive) (reviewed in Parsons, 1990). Mutations at this locus were also identified as *sextra* (Rogge et al., 1992)and *multiple inner photoreceptors* (Buckles et al., 1992). Loss-of-function and reduction-of-function mutations at this locus dominantly suppress the phenotype associated with a very weak allele of *sevenless* and recessively suppress the phenotype of strong alleles of *sevenless*(Buckles et al., 1992, Gaul et al., 1992, Rogge et al., 1992). Apparent null mutations at this locus are viable (Buckles et al., 1992, Gaul et al., 1992, Rogge et al., 1992), suggesting that Gap activity is either redundant or dispensable for viability. Animals homozygous for these mutations differentiate excessive R7-like cells (Buckles et al., 1992, Gaul et al., 1992, Rogge et al., 1992). This gene product is autonomous to the ectopic R7 cells, as wild-type activity in these cells is sufficient to prevent their development as presumptive R7 cells (Buckles et al., 1992, Gaul et al., 1992, Rogge et al., 1992). This gene is not apparently required for the proper development of the other photoreceptor cells, as the other photoreceptor cell types appear wild-type. Mutations at this locus, while able to recruit ectopic R7 cells, are unable to make the presumptive R7 cell *sevenless* independent (Buckles et al., 1992, Rogge et al., 1992). This is possibly due to either temporal restrictions, weak mutations, or the independence of the *sevenless* - mediated pathway from Gap1 activity. This gene probably acts in the DER pathway also, although no data has been presented to show that this is the case.

***drk*, a GRB2 homolog, couples receptors and a guanine nucleotide exchange factor**

downstream of receptor kinase (drk), a homolog of the *sem-5 / GRB2* gene product has been identified as an enhancer of a weak *sevenless* allele and a suppressor of a *sevenless* gain-of-function allele (Olivier et al., 1993, Simon et al., 1993). These mutations confer a lethal phenotype when homozygous (Simon et al., 1993), suggesting a broader role for this gene than just interaction with *sevenless*. These mutations also interact with mutations at DER (Simon et al., 1991). In *vitro*, a physical association is observed between the SH2 domain of *downstream of receptor kinase* and the C-terminal tail of both *sevenless* (Olivier et al., 1993, Simon et al., 1993) and the human EGF receptor (Olivier et al., 1993, Simon et al., 1993) and *downstream of receptor kinase (drk)* and the C-terminus of the *Son of sevenless* gene product (Olivier et al., 1993, Simon et al., 1993),. *Son of sevenless* encodes a putative guanine nucleotide exchange factor (GNEF) (Bonfini et al., 1992). This interaction suggests the mechanism of the control of *ras* activity via receptor tyrosine kinases. The receptor tyrosine kinase autophosphorylates, creating a *sem-5 / GRB2 / drk* binding site. This binding subsequently either allows the binding of *sem-5 / GRB2 / drk* to *Son of sevenless* or brings the bound *Son of sevenless* to the receptor tyrosine kinase, increasing the ability of *Son of sevenless* to activate nucleotide exchange at *ras*. This class of protein may play no catalytic role aside from enabling the regulated association of proteins.

***Son of sevenless* encodes a guanine nucleotide exchange factor, a regulator of *ras* activity**

Another regulator of *ras* has been identified as acting in both the *sevenless* and DER signal transduction pathway. The *Son of sevenless* locus encodes a putative guanine nucleotide exchange factor (Bonfini et al., 1992). This class of molecules is believed to act to promote the conversion of *ras*-GDP to *ras*-GTP by promoting the release of bound GDP and the subsequent binding of *ras* to GTP, resulting in the activation of *ras* (Downward, 1992). Both dominant and recessive mutations at this locus have been identified (Rogge et al., 1991). Dominant mutations at this locus result in allele-specific suppression of *sevenless* mutations (Rogge et al., 1991). This suppression is sensitive to the dosage of several genes required for the development of R7, including *Son of sevenless* itself, *bride of sevenless* (the putative *sevenless* ligand), *Gap1*, and *Ras1* (Rogge et al., 1991), suggesting that all of these genes act in the same pathway. The *Son of sevenless* dominant mutations confer no phenotype other than suppression of the *sevenless* phenotype (Rogge et al., 1991). This suppression is autonomous to the presumptive R7 cell (Rogge et al., 1991). Loss-of-function mutations at the *Son of sevenless* locus result in lethality (Rogge et al., 1991). Examination of rare viable animals bearing two reduction-of-function alleles at this locus suggest that this gene is required for the development of R7 and some of the other photoreceptor cells (Rogge et al., 1991). Mutations at the *Son of sevenless* locus also interact with the *Ellipse* mutation of DER. Dominant mutations at *Son of sevenless* enhance the phenotype of *Ellipse*, while loss-of-function alleles dominantly suppress the *Ellipse* phenotype (Rogge et al., 1991), implicating this gene in transduction through DER. This is also consistent with the interaction of *Son of sevenless* and *drk*. *drk* binds to the human EGF receptor (Olivier et al., 1993), and mutations at *drk* interact with *Ellipse* alleles (Simon et al.,

1993), suggesting that *drk* acts to transduce signal from DER as well as *sevenless*.

Ras1 acts in DER transduction

Mutations at the *Ras1* locus also enhance the phenotype of a weak allele of *sevenless* and enhance the phenotype of the *Ellipse* mutation (Simon et al., 1993). Deletions of this locus enhance this phenotype as well (Simon et al., 1993). Animals bearing loss-of-function mutations at this locus die prior to eye differentiation. Therefore these animals have not been analyzed for the role of *ras* in the development of other cells in the eye. Animals bearing activated *ras* transgenes display *sevenless* independent development of R7 cells (Fortini et al., 1992). It is not clear whether either *bride of sevenless* or *sevenless* wild-type activity is required for the development of the "true" R7 cell in these animals (the cell which is properly positioned to become R7). In these mutant backgrounds, other cells can be recruited to assume an R7-like fate (Simon et al., 1991), but it has not been confirmed whether these animals have eyes similar to the eyes of double mutant *sevenless/sevenless; Gap1/+* animals. In these (*sevenless/sevenless; Gap1/+*) animals, the presumptive R7 differentiates as a non-photoreceptor, while other cells are recruited to become R7 cells (Buckles et al., 1992, Rogge et al., 1992). Since Gap1 mediates the activity of the *Ras1* gene product, it is likely that the phenotypes associated with these mutations would be similar. However, it seems clear that *ras* acts as a switch gene in the *sevenless* signaling pathway, much as it does in the vulval differentiation pathway of *C. elegans*.

raf may act in DER mediated transduction as well

Genetic and molecular experiments implicate the *Drosophila* raf serine/threonine kinase in the *sevenless* pathway of eye development (Dickson et al., 1992). Mutations at *l(1)polehole*, the locus encoding *raf*, generally cause inviability (Nishida et al., 1988). When rare viable animals are observed, their eyes are abnormal, displaying failures on the part of the R7 cell as well as other photoreceptors to properly differentiate (Dickson et al., 1992). Transgenic animals carrying putative activated Raf constructs display the opposite phenotype; ectopic R7 cells differentiate (Dickson et al., 1992). This *raf* gain-of-function phenotype is epistatic to the a *sevenless* mutation, and a *raf* mutation suppresses the phenotype associated with a *sevenless* gain-of-function allele (Dickson et al., 1992), suggesting that Raf acts after *sevenless* in the development of the R7 cell. Further analysis of doubly and triply mutant animals suggest that *raf* may act after *ras* as well, since animals bearing mutations at *sevenless, raf,* and an activated *ras* transgene display a phenotype most similar to that of the *raf* mutation (Dickson et al., 1992). This gene has not been shown to act in the DER pathway yet, but it seems likely, as it acts after *ras* in the eye and *ras* acts after DER as well as *sevenless*. Implication of *raf* in the DER pathway will be consistent with the activity of *lin-45 raf* in transduction through *let-23*.

argos/giant lens encodes a secreted negative regulator

One gene that acts in a manner analogous to that of *lin-15* has been identified in the development of the *Drosophila* eye. Mutations at this locus (*argos* or *giant lens*) result in the recruitment of extra cells to assume the photoreceptor fate (Freeman et al., 1992, Kretzschmar et al., 1992). Mosaic analysis of mutations at this locus suggest that, like *lin-15*, this gene acts

non-autonomously (Freeman et al., 1992, Kretzschmar et al., 1992). However, it is not known whether this gene interacts at all with the *sevenless* mediated pathway of photoreceptor differentiation, or whether any of the excess photoreceptors are apparent R7 cells. Analysis of the non-autonomous negative regulators of receptors (such as *lin-15* and *argos*) will add a further layer of understanding to the control of receptor activity. It would be interesting to know if this mutation results in cells which express *sevenless* but are not exposed to *bride of sevenless* differentiating as R7. If this is true, then this gene may act in a manner analogous to *lin-15*. Since the ligand for DER is not conclusively known, such analysis with respect to DER would not be possible yet.

Thus, in *Drosophila* as in *C. elegans,* an EGF receptor mediated signal transduction pathway has been characterized genetically. Several genes have been identified in both *Drosophila* and *C. elegans*. The screens for modifiers of *sevenless* mutations have allowed the identification of several of these genes. It will be interesting to see what other loci can be mutated to suppress *sevenless* mutations. One might expect that the a *sli-1* like activity may function in this pathway, however, it is also possible that such a gene may act as a modifier of DER only, and thus would not be identified as a modifier of *sevenless*. Similarly, one might expect that a *Drosophila* homolog of *unc-101* might be identified as a modifier of *sevenless*, especially since internalization is required for transduction via the *sevenless / bride of sevenless* complex (Cagan et al., 1992).

Mammalian Systems

Mammalian EGF receptor signal transduction

In mammals, there are at least four homologs of the EGF receptor, although only a single receptor responds to EGF. The other receptors are architecturally similar in their general structure and presumably interact with a similar set of partners. I will concentrate on the EGF receptor, as this is the best characterized member of this family. Most of the work on these receptors has utilized tissue culture techniques rather than in *vivo* studies. Several cell lines that overexpress the EGF receptor have been characterized. Other EGF receptor negative cell lines are capable of responding to EGF if the receptor is present on a transgene.

EGF receptor apparently works through homologs of all of the genes listed above (EGF, GRB2, GNEF, ras, GAP, and raf) as well as a number of other genes (phospholipase C-γ, phosphotidylinositol-3 [PI3] kinase, protein kinase C, and mitogen activated protein [MAP] kinase and an unknown calcium dependent kinase) (reviewed in Carpenter and Cohen, 1990, Chao, 1992, Schlessinger and Ullrich, 1992) not yet implicated in either *C. elegans* or *D. melanogaster*. The mechanism of the activation of the receptor is reviewed in (Schlessinger and Ullrich, 1992). In brief, EGF receptor first binds EGF. This allows dimerization of receptor. Upon dimerization the receptors either autophosphorylate or *trans*-phosphorylate their partner. This autophosphorylation is essential for the interaction of the stimulated receptor with the SH2 domains of its downstream targets. The eventual results of this cascade include DNA replication, changes in transcription, changes in cell morphology, and desensitization to further EGF addition. The role of some of the target proteins is unclear. PI3 kinase is believed to act in signal

transduction by affecting intracellular protein targeting (Schu et al., 1993). Phospholipase C-γ (PLC-γ) and protein kinase C are activated by stimulation of the EGF receptor (see below). MAP kinase acts as a positive regulator of EGF receptor (Countaway et al., 1989) and may be one of the last targets of this cascade. One would expect that homologs of these genes will soon be identified in the invertebrate systems and may already exist as uncloned but genetically identified loci. Upon addition of EGF in tissue culture experiments, several of these proteins associate with the EGF receptor, and these and other proteins are phosphorylated, presumably by the EGF receptor and by other kinases activated by the EGF receptor (reviewed in (Carpenter and Cohen, 1990, Chao, 1992, Schlessinger and Ullrich, 1992)). The role of this phosphorylation in the activity of these proteins is variable. Some of the targets are apparently activated by phosphorylation, while others do not seem to become more active. The activation of these proteins probably involves more parameters than phosphorylation. Protein interactions are probably essential and such interactions are difficult to duplicate in vitro.

Phosphotidylinositol 3'-kinase

PI3 kinase is one of the substrates of EGF receptor that is found associated with the receptor in EGF stimulated cells. This enzyme catalyzes the phosphorylation of $PI4P_2$ to $PI(3,4)P_3$ and $PI(4,5)P_2$ to $PI(3,4,5)P_3$. This protein consists of two subunits, the regulatory p85 subunit and the catalytic p110 subunit. The interaction between the PI3 kinase complex and the EGF receptor is presumably mediated by the SH2 domains of the p85 subunit binding to a subset of the phosphotyrosines of the EGF receptor (Koch et al., 1991). Upon EGF stimulation, the PI3 kinase activity recoverable by

immunoprecipitation using an anti-phosphotyrosine antibody increases (Carter and Downes, 1992). PI(3,4) P_2 and PI(3,4,5)P_3 levels increase as well (Carter and Downes, 1992, Payrastre et al., 1991). PI(3,4,5)P_3 stimulates the ζ isozyme of Protein Kinase C (Nakanishi et al., 1993). A deletion of the yeast PI3 kinase p110 results in a protein-sorting defect (Schu et al., 1993). Since activity of the EGF receptor is regulated at least partially via controlling receptor recycling through the vacuole and/or degradation within the endosome, it is interesting to speculate on the roles of PI3 Kinase and AP47 (*unc-101*, a regulator of *let-23*, encodes the *C. elegans* homolog of the mammalian *trans*-Golgi associated clathrin adaptor medium chain AP47, see chapter 3). Perhaps both PI3 kinase and *unc-101* are controlling the ability of the endosome to regulate the degradation and recycling of the EGF receptor and EGF, or affect the ability of the EGF/EGF receptor complex to transduce signal from within the endosome.

Phospholipase C-γ

Phospholipase C-γ associates with the EGF-stimulated EGF receptor. PLC-γ is one of the targets of EGF receptor tyrosine kinase activity. This enzyme acts to enzymatically produce the second messengers diacylglycerol and Inositol 3 phosphate (IP3), which activate protein kinase C and causes the release of intracellular calcium stores. Tyrosine phosphorylation of PLC-γ increases upon addition of EGF to cells expressing the EGF receptor (Meisenhelder et al., 1989, Wahl et al., 1989). Recent experiments have shown that this phosphorylation increases the activity of PLC-γ (Nishibe et al., 1990). Phospholipase C-γ interacts with the autophosphorylated EGF receptor by virtue of the PLC-γ SH2 domain and autophosphorylated

tyrosines of the EGF receptor (Rotin et al., 1992, Vega et al., 1992). However, the tyrosine in the receptor (Y992) that interacts with the PLC-γ SH2 domain in *vitro* has never been shown to be a site of autophosphorylation in *vivo* or in *vitro* in a wild-type receptor . If much of the C-terminus is truncated (Helin et al., 1991, Walton et al., 1990), or the four major sites of autophosphorylation are mutated to phenylalanine (Sorkin et al., 1992) this residue (Y992) becomes an autophosphorylation site. Mutation of this tyrosine only in a wild-type receptor does not decrease internalization of the EGF receptor or tyrosine phosphorylation of PLC-γ (Sorkin et al., 1992). The mechanism of the EGF receptor/ PLC-γ interaction remains unclear. PLC-γ indirectly controls one, and possibly more, of the negative regulators of the EGF receptor, as activation of PLC-γ results in the formation of diacylglycerol (an activator of protein kinase C) and IP3, which triggers calcium release, potentially activating calcium dependent protein kinase II.

Protein Kinase C

Protein kinase C is a serine/threonine kinase activated by PLC-γ. This kinase likely functions as a negative regulator of the EGF receptor (and may also act to transduce the signal). Upon addition of EGF, protein kinase C activity increases. Treatment of EGF receptor expressing cells with phorbol esters (which activate protein kinase C) results in the down regulation of the ability of these cells to bind and respond to EGF. This is similar to the down regulation of the EGF receptor in response to prolonged EGF treatment. This down regulation will be discussed further in the following sections.

MAP Kinase

Several downstream kinases have been characterized as actors in EGF mediated signal transduction. One of these is MAP Kinase. This serine/threonine kinase likely acts to transduce the EGF signal and may be a direct or indirect target of the *raf* serine/threonine kinase. MAP kinase has been identified as a kinase that phosphorylates the EGF receptor after addition of EGF (Countaway et al., 1989). This phosphorylation event appears to increase the activity of the EGF receptor (Countaway et al., 1989). The targets of this kinase may include transcription factors(Marais et al., 1993).

Mullerian Inhibiting Substance

Mullerian Inhibiting Substance (MIS) is functionally similar to *lin-15* and *argos*. MIS is secreted. Addition of MIS prevents EGF receptor autophosphorylation (Cigarro et al., 1989). MIS does not compete with EGF for a binding site on the EGF receptor (Cigarro et al., 1989) and may not interact directly with the EGF receptor.

Mechanisms of EGF receptor down regulation

Upon addition of EGF, the EGF receptor is the target of a number of negative regulatory loops. The end result of the combination of these processes is that the cell becomes far less sensitive to EGF, despite retaining significant amounts of the EGF receptor on the surface of the cell, These processes include (1) internalization and degradation, phosphorylation at (2) threonine 654 (T654), (3) serine 1046/7 (S1046/7), (4) regulation by a Ca^{++} sensitive kinase, and (5) dephosphorylation of C-terminal phosphotyrosines. These processes combine to rapidly desensitize EGF treated cells to further

response to EGF.

Internalization and degradation

Internalization and subsequent degradation is the major pathway of EGF receptor down regulation. Upon EGF binding, the EGF/EGF receptor complexes collect in coated pits and are internalized (Haigler et al., 1978, Schlessinger et al., 1978). This process is saturable on cells expressing large amounts of the receptor treated with large amounts of ligand (Gilligan et al., 1992, Gilligan et al., 1990, Lund et al., 1990, Wiley, 1988), suggesting that the cellular endocytic machinery is limiting in these cells.

Kinase activity may be required for internalization. The data from these experiments conflicts, as different experimental regimens have been followed. Most experiments have shown that "kinase inactive" EGF receptors do not undergo internalization upon addition of EGF (Glenney et al., 1988, Lund et al., 1990, Masui et al., 1991, Prywes et al., 1986, Wiley et al., 1991). Other experiments have shown that a "kinase inactive" receptor internalizes (Chen et al., 1989, Felder et al., 1992, Honegger et al., 1987) but is not down-regulated (Felder et al., 1992, Honegger et al., 1987). Two hypotheses have been proposed: that kinase activity is required for internalization or that kinase activity is required for degradation or routing within the cell. These experiments were performed with different mutations in different cell lines treated with different concentrations of EGF and different statistical analyses. The regulation of internalization may vary by cell type or some of these cells may express enough of the endogenous receptor to deliver measurable amounts of the receptor to the coated pits. The C-terminal tail of the receptor has been shown to be involved in the regulation of

internalization. Deletions of the residues C-terminal to the tyrosine kinase domain results in a receptor molecule that is no longer internalized following stimulation with EGF (Chang et al., 1991, Chen et al., 1989, Felder et al., 1992, Li et al., 1991). A small (18 amino acid) stretch (the cain domain) is essential for internalization as well as calcium release (Chang et al., 1991, Chen et al., 1989). This site is near the residues which make up the putative PLC-γ binding site (Rotin et al., 1992, Vega et al., 1992). If the three major autophosphorylation sites in the C-terminal tail of the receptor are mutationally inactivated, internalization is decreased (Helin and Beguinot, 1991, Sorkin et al., 1992, Sorkin et al., 1991). Mutation of two of these residues does not prevent internalization (Helin and Beguinot, 1991). These mutations did not alter the tyrosine which is present in the putative PLC-γ binding site. .These experiments suggest that C-terminal regions and phosphorylation of residues in this region are important for the regulation of internalization. Phosphorylation on the serine residues at S1046/7 is also important for the regulation of internalization. If these serines are mutated to alanines, down-regulation is impaired (Countaway et al., 1992, Theroux et al., 1992). Several regions of the EGF receptor are required for down-regulation. Some of the proteins acting to regulate this process have been identified, but many others are as yet unknown.

Kinase activity of the receptor may act to stabilize the association of the receptor with the endocytotic machinery (Lai et al., 1989), suggesting that some of the variation seen in these experiments may reflect variation in the ability of the endocytosis machinery to associate with the receptor in the absence of kinase activity. Some experiments have shown that autophosphorylation is suppressed until the receptor enters the endocytotic

pathway but increases in the vesicles (Lai et al., 1989, McCune et al., 1990).

The fraction of wild-type receptor that is recycled to the surface after internalization also varies with tissue, ligand, and kinase activity. As mentioned earlier, some kinase inactive receptors recycle completely (Felder et al., 1992, Honegger et al., 1987), while others do not (Glenney et al., 1988, Lund et al., 1990, Masui et al., 1991, Prywes et al., 1986, Wiley et al., 1991). In some tissues, a great deal of the receptor recycles (Dunn et al., 1986, Jackle et al., 1991, Murthy et al., 1986, Sorkin et al., 1991), while in other tissues very little of the receptor recycles(Lai et al., 1989). Degradation of internalized ligand is generally more complete than degradation of internalized receptor. Transforming Growth Factor α (TGF-α) apparently induces less degradation than does EGF (Ebner and Derynck, 1991). Regulation of recycling may be a biologically relevant method of controlling the sensitivity of cells to EGF.

Protein Kinase C and threonine 654

One of the other modes of desensitization of EGF receptor is phosphorylation at threonine 654 (T654) (Hunter et al., 1984). Upon addition of EGF, there is a transient increase in the fraction of receptor molecules phosphorylated at this residue (Decker et al., 1990, King and Cooper, 1986, Whiteley and Glaser, 1986). This residue is a target of protein kinase C (Hunter et al., 1984, Whiteley and Glaser, 1986). Protein kinase C is activated indirectly by the EGF receptor (as well as many other kinases) via the activation of phospholipase C-γ (PLC-γ). The activation of PLC-γ results in the hydrolysis of PIP2 into DAG and IP3. DAG directly activates protein kinase C, and IP3 induces the release of intracellular calcium, which also

results in the activation of protein kinase C. Phorbol ester treatment activates protein kinase C, leading to extensive phosphorylation of the EGF receptor at this residue (Cochet et al., 1984, Downward et al., 1985, Iwashita and Fox, 1984), Davis, 1985 #938. If cells are first treated with phorbol ester and subsequently challenged with EGF, there is very little response to EGF (Bowen et al., 1991, Cochet et al., 1984, Countaway et al., 1990, Davis, 1988, Downward et al., 1985, Felder et al., 1992, Lund et al., 1990). This is similar to the down regulation seen when cells are pretreated with EGF rather than phorbol esters. This down regulation includes decreased affinity for EGF (Davis, 1988, Downward et al., 1985, Felder et al., 1992), decreased tyrosine kinase activity (Friedman et al., 1984), Cochet, 1984 #970; Davis, 1988 #937; Lund, 1990 #980, and a decrease in the internalization of receptor in response to the addition of EGF (Lund et al., 1990) Substitution of threonine 654 with alanine (T654A) renders cells expressing this mutant EGF receptor independent of phorbol ester, as phorbol ester pretreatment no longer desensitizes these cells to EGF treatment (Davis, 1988, Decker et al., 1990, Lin et al., 1986, Lund et al., 1990). Mutation of this residue to tyrosine gives similar results (Livneh et al., 1988). Mutation of this residue to serine (T654S) results in constitutive phosphorylation (Bowen et al., 1991). This mutation decreases the mitogenic response to EGF treatment (Bowen et al., 1991). The C-terminal region, as mentioned earlier, is involved in down regulation via internalization. In response to a pulse of EGF, cells expressing wild-type EGF receptor undergo transient morphological changes, briefly releasing from the substrate and altering their cytoskeleton. C-terminal deletions increase the persistence of these morphological changes (Welsh et al., 1991). Cells expressing EGF receptor with both C-terminal deletions and

the T654A mutation display an even greater response to this stimulation. These cells undergo a more pronounced response to this signal and take longer to recover to the normal morphology (Welsh et al., 1991). These mutated (T654A, C-terminal deleted) receptors display a heightened resistance to desensitization by phorbol ester treatment compared to wild-type or singly mutated receptors (Lund et al., 1990). Negative regulation by protein kinase C appears to be less important than down regulation of the receptor by internalization, as the T654A mutation in a wild-type receptor is not sufficient to block negative regulation of an otherwise wild-type receptor (Lin et al., 1986). Cells which express receptors with the T654A mutation are more responsive to EGF treatment than are cells expressing wild-type receptors (Decker et al., 1990, Welsh et al., 1991). Thus, this is a biologically relevant negative regulation loop, which may be a short term negative regulation (Decker et al., 1990) as opposed to the slower internalization. T654S, a mutation at the Protein Kinase C site resulting in constitutive phosphorylation at this residue by Protein Kinase C is unable to transduce signal (Bowen et al., 1991). Phosphorylation at this residue is not sufficient to explain negative regulation of the receptor, as only a small fraction of receptors are phosphorylated on this residue and this phosphorylation is transient (Bowen et al., 1991).

Serine 1046/7

A pair of residues within the receptor that appear to function in the negative regulation of receptor signaling are serine 1046/7. These residues are major sites of phosphorylation of the EGF receptor (Countaway et al., 1990, Heisermann and Gill, 1988). These residues are in *vitro* target of

calcium dependent protein kinase II (Countaway et al., 1992), but it has yet to be shown that this kinase phosphorylates the EGF receptor in *vivo*. Phosphorylation at this residue decreases the ability of the receptor to autophosphorylate (Countaway et al., 1992), suggesting that the role of phosphorylation at this residue is negative regulation of the receptor. Mutation of these serines to alanines (S1046/7A) results in less desensitization to EGF (Countaway et al., 1992, Theroux et al., 1992). These S1046/7A mutant receptors fail to internalize in response to EGF binding (Countaway et al., 1992, Theroux et al., 1992) and display increased tyrosine kinase activity in response to EGF stimulation (Countaway et al., 1992, Theroux et al., 1992). This may be one of the major residues in the C-terminus required for internalization. This residue is near one of the tyrosines (Y1048) that is a major target of autophosphorylation and this proximity may be important in the regulation of autophosphorylation activity or of interaction of this phosphotyrosine with SH2 domains.

Calcium regulation

One of the effects of the transduction of the EGF mediated signal via the EGF receptor is the release of intracellular calcium (Kuppuswamy and Pike, 1989, Lin et al., 1986). One region of the EGF receptor has been shown to be required for EGF stimulated calcium release. This region is 18 amino acid residues (Chang et al., 1991, Chen et al., 1989) near the putative binding site for the indirect regulator of calcium release, PLC-γ(Rotin et al., 1992, Vega et al., 1992). Treatment with calcium ionophore results in down regulation of the EGF receptor (Friedman et al., 1989, Kuppuswamy and Pike, 1989). Since calcium release results in the down regulation of the receptor, calcium

may play a biological role in the down regulation of the EGF receptor. Two of the candidates for calcium regulation are protein kinase C and calcium dependent protein kinase II. Calcium dependent protein kinase II phosphorylates the EGF receptor in *vitro* (Countaway et al., 1992), and protein kinase C has been shown to act on the EGF receptor in *vivo* (Hunter et al., 1984) Both of these kinases phosphorylate specific residues on the EGF receptor (Countaway et al., 1992, Hunter et al., 1984). This phosphorylation decreases the tyrosine kinase of the receptor (Cochet et al., 1984, Countaway et al., 1992, Davis, 1988, Lund et al., 1990) and increases down-regulation of the receptor (Countaway et al., 1992, Lund et al., 1990, Theroux et al., 1992), Davis, 1988 #937; Livneh, 1988 #957. If this is the biological kinase or similar to the biological kinase, then PLC-γ is indirectly controlling at least two negative feedback loops.

Threonine 669

The major EGF stimulated threonine phosphorylation site is threonine 669 (T669). This site is apparently the target of two MAP Kinases (Countaway et al., 1989, Northwood et al., 1991), which are themselves presumably indirect targets of the Raf kinase (Kyriakis et al., 1992). Phosphorylation at this residue is increased by treatment with phorbol esters (Countaway et al., 1989), suggesting that protein kinase C activates this MAP Kinase activity as well. However, phosphorylation at T669 does not appear to play a major role in the regulation of the sensitivity of the EGF receptor. Mutation of this threonine to alanine (T669A) does not interrupt internalization or down regulation (Countaway et al., 1989). Cells bearing this mutation are less mitogenic in response to EGF. The mutation of this

residue as well as serine 671 results in a decrease in internalization with no noticeable effect on autophosphorylation (Heisermann et al., 1990), suggesting that these two residues redundantly act in the control of internalization. This doubly mutant receptor can still be down regulated by treatment with phorbol ester (Heisermann et al., 1990), suggesting that phosphorylation by protein kinase C is sufficient to direct internalization in the absence of phosphorylation at these residues. Mutation of these two residues also decreases the amount of the regulatory PI3 kinase subunit, p85, which is bound to activated receptor (Heisermann et al., 1990). These two residues, in combination, may act as a region controlling negative regulation, potentially by regulating the interaction with PI3 kinase. Alternatively, the serine 671 mutation may be the cause of this defect in negative regulation.

Endosome-associated phosphotyrosine phosphatase

A novel membrane bound phosphatase has been identified that dephosphorylates C-terminal tyrosines of the EGF receptor (Faure et al., 1992). This activity is tightly associated with the endosomal membrane (Faure et al., 1992). Very little is known regarding this candidate negative regulator. It is not one of the characterized membrane tyrosine phosphatases. It is not known whether the activity of this molecule is regulated or is biologically relevant in the desensitization of the EGF receptor upon addition of EGF.

Redundancy of negative regulation in vertebrates and invertebrates

Biochemistry and molecular genetics have defined a number of pathways of negative regulation acting on the EGF receptor. It appears that these pathways are redundant, in that mutations which prevent or reduce the ability of one of them to function do not result in a receptor which does not undergo down regulation. Instead, these mutated receptors are partially defective in down regulation, often displaying relatively small increases in the response to EGF or partial defects in down regulation. Given the redundancy of these pathways, it is perhaps not surprising that the genes identified as suppressors of *let-23* do not cause defects in vulval differentiation in an otherwise wild-type background. These suppressors do cause defects in regulation when combined, suggesting that removing two of the pathways of negative regulation may be sufficient to significantly impact the activity of the receptor. This is paralleled nicely by the experiments examining morphological changes in cells bearing EGF receptor with C-terminal deletions and/or the T654A mutation (Decker et al., 1990, Welsh et al., 1991). Here too, cells bearing receptors which have been mutated to disable two pathways of negative regulation display greater responsiveness to EGF than that seen in cells bearing either mutation alone. The combination of genetic and biochemical approaches to the negative regulation of EGF receptor will shed light on the mechanisms underlying this important phenomenon.

Relevance

I have used a genetic approach to characterize negative regulators of the EGF receptor homolog encoded by *let-23*. Genetically identified regulators of this gene may prove to act as regulators of the EGF receptor in other species,

including mammals. The study of both biochemically and genetically characterized negative regulators will increase the understanding of the process of desensitization of the EGF receptor. Identification of additional genes (see appendix) acting as negative regulators of vulval differentiation will allow further characterization of negative regulation of *let-23* and the molecular characterization of these genes in *C. elegans* and other species will show how conserved these processes are.

Figure 1. The EGF mediated signal transduction pathway in *C. elegans, Drosophila melanogaster* and mammals. Abbreviations: DER, *Drosophila* Epidermal Growth Factor receptor; GAP and GAP1, GTPase activating proteins; and GNEF, guanine nucleotide exchange factor; MAPK, mitogen activated protein kinase.

Figure 1. EGF mediated signaling in diverse systems

	C. elegans	*Drosophila*	mammal
LIGAND	*lin-3*	*spitz* + ?	EGF + TGF-α
RECEPTOR	*let-23*	DER	EGF-r
GAP	?	*Gap1*	GAP
SH2-SH3-SH2	*sem-5*	*drk*	GRB2
GNEF	?	*Son of sevenless*	CDC25
ras	*let-60*	*Ras1*	*ras*
raf	*lin-45*	*l(1)polehole*	*raf*
MAPK	?	?	MAPK

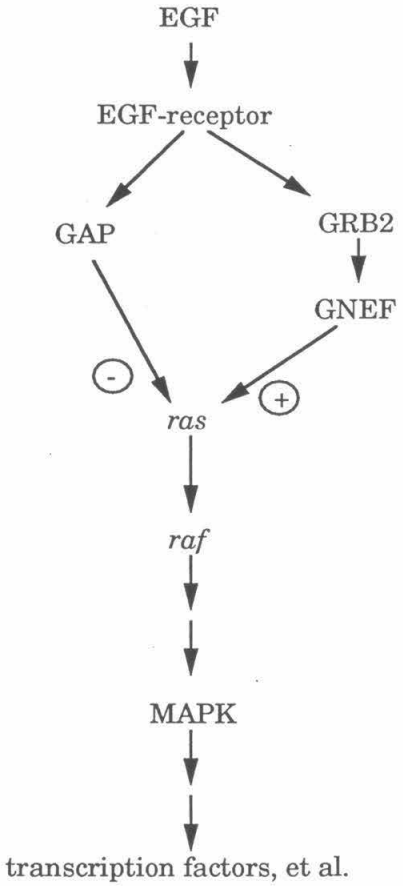

Figure 2. Vulval lineages and regulation. See text. L denotes a longitudinal (anterior/posterior) axis of division, T denotes a transverse axis of division, and N denotes no division. All divisions are on an anterior/posterior axis unless noted.

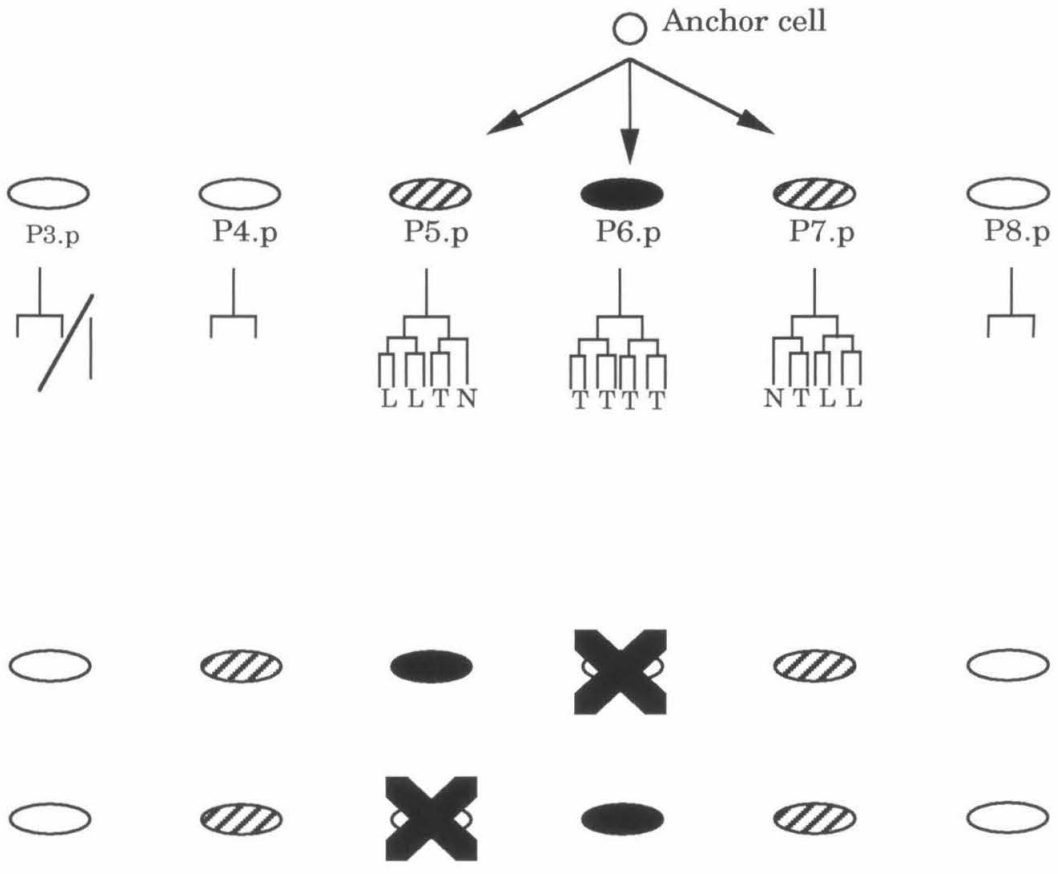

Figure 2. Vulval lineages and regulation

Figure 3. Structure of the *let-23* Epidermal Growth Factor receptor tyrosine kinase compared to the mammalian EGF receptor. C-rich denotes cysteine rich regions, TM denotes transmembrane domain, juxta denotes the juxtamembrane region. The second C-rich domain of *let-23* is larger than that of other family members. T654 is located in the juxtamembrane region, Y992 and S1046/7 reside in the tail region of the EGF receptor.

Epidermal Growth Factor receptor

let-23

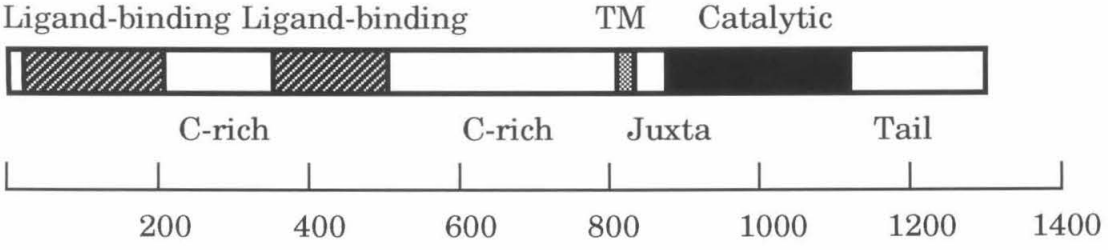

Figure 3

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Chapter 2

Genetic characterization of *sli-1*

Prepared for submission to Genetics

B-2

sli-1, a silent suppressor of weak mutations at the *C. elegans let-23* locus, is apparently a negative regulator of *let-23*

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ABSTRACT

Mutations at the *sli-1* (suppressor of lineage defect) locus suppress the lineage defects associated with mutations at a number of genes required for proper vulval differentiation in *C. elegans*. Mutations at this locus are otherwise silent. *sli-1* mutations suppress all phenotypes associated with hypomorphic alleles of *let-23* but do not suppress null alleles of *let-23*, suggesting that a *sli-1* mutation does not bypass the requirement for functional *let-23*. Mutations at *sli-1* suppress the vulval defects but not other defects associated with mutations at *sem-5*, a gene believed to interact with *let-23* in the vulva. This locus displays dosage sensitivity in some genetic backgrounds. *sli-1* is an apparent negative regulator of vulval differentiation, potentially acting at or near the *let-23* step.

INTRODUCTION

The process of vulval induction in *C. elegans* is a well studied example of proto-oncogene mediated signal transduction. The anchor cell (AC) of the somatic gonad produces an inductive signal (Kimble, 1981). This signal, which is encoded by the *lin-3* gene, is similar to Epidermal growth factor (EGF) (Hill and Sternberg, 1992). In response to this signal, the Vulval Precursor Cells (VPCs) form vulval tissue. This response is presumably mediated by *let-23*, an EGF receptor homolog (Aroian et al., 1990), *lin-10* (Kim and Horvitz, 1990), *sem-5*, a SH2/SH3 *grb-2* homolog (Clark et al., 1992, Lowenstein et al., 1992), *let 60 ras* (Han and Sternberg, 1990) and *lin-45 raf* (Han et al., 1993). Reduction of function mutations at any of these loci cause mos or all of the VPCs to remain uninduced and form hypodermal tissue. Thus, an animal homozygous for one of these mutations is Vulvaless (Vul, Fig. 1). Little is known about the negative regulation of this process. The *lin-15* gene functions from the surrounding hypodermal syncytium to prevent signal independent activation of vulval fates (Ferguson et al., 1987, Herman and Hedgecock, 1990). *lin-1* is required for hypodermal fates but is epistatic to all of the Vul mutations, and therefore acts at a very late step in the cascade (Ferguson et al., 1987, Han et al., 1993, Han and Sternberg, 1990, Herman and Hedgecock, 1990) (G. Beitel, S. Clark, and R. Horvitz, pers. comm.). In the absence of either the *lin-1* or *lin-15* gene products, all of the VPCs assume vulval fates in a signal independent manner, resulting in the formation of ventral pseudovulvae (the Multivulva or Muv phenotype, Fig. 1). Three genes have been implicated in the negative regulation of the response to the inductive signal.

These genes, *let-23, lin-2* and *lin-7*, are required for response to the inductive signal as well as negative regulation of this response. Jongeward and Sternberg, manuscript in preparation. Rare alleles of these loci display hypersensitivity to the inductive signal. This is reflected in the Hyperinduced or Hin phenotype (Fig. 1), which is defined as a greater than wild-type signal dependent vulval differentiation. This may reflect a defect in the ability of these gene products to properly negative-regulate the response to the signal

let-23 functions in several tissues throughout *C. elegans* development (Aroian and Sternberg, 1991). This locus has been extensively characterized genetically (Aroian and Sternberg, 1991) and molecularly (Aroian et al., 1990). It would be of interest to define genes that function to regulate the activity or expression of *let-23*. To this end, we have recovered and characterized mutations that suppress weak mutations of *let-23*. The screens used to recover these suppressors were designed to identify 1) intragenic revertants, as well as loci which 2) transduce the inductive signal after *let-23*, 3) interact with *let-23*, or 4) are involved in negative regulation of the response to the inductive signal. This approach was successful, as we have identified mutations of at least three of the expected classes. We identified an intragenic revertant of *let-23* which restores a nearly wild-type phenotype. One *let-60ras(gf)* allele was recovered. This mutation is epistatic to a *let-23* mutation in the vulva. We also recovered mutations at loci which appear to function as negative regulators of *let-23*. Here we describe our analysis of one of these loci, *sli-1* (suppressor of

lineage defect). *sli-1* may function as a negative regulator of *let-23*, although it is not clear whether this is a direct or indirect interaction.

Mutations at the *sli-1* locus confer no obvious phenotype in an otherwise wild-type background, but cause animals which are homozygous for both a *sli-1* mutation and a homozygous viable mutation in *let-23, lin-2, lin-7, or lin-10* to differentiate excessive vulval tissue in a signal dependent manner (the Hyperinduced or Hin phenotype, Fig. 1). Mutations at the *sli-1* locus are sufficient to suppress *let-23* defects in all of the tissues in which *let-23* is known to function, suggesting that the *sli-1* gene product interacts with *let-23* in all of the developmental decisions in which *let-23* is involved. *sli-1* mutations do not suppress the lethality associated with strong alleles of *let-23, lin-3, let-60* or *lin-45*, suggesting that *sli-1* does not function as a late-acting gene required for the hypodermal (uninduced) fate which these genes antagonize. Thus, in at least one tissue, a *sli-1* mutation is insufficient to bypass the requirement for functional *let-23*. In contrast, a *let-60 ras(gf)* mutation partially relieves the requirement for functional *let-23* for both the essential function and the vulval function (Han and Sternberg, 1990). The ability of *sli-1* to suppress *let-23* mutations requires some *let-23* gene product which is partially functional. Thus, *sli-1* seems to function as a negative regulator which is distinct from the main trunk of the signal transduction pathway, as defined by *lin-3, let-23, sem-5, let-60,* and *lin-45*. We argue that *sli-1* may function as a negative regulator of *let-23* (rather than one of the other genes in the pathway) for three reasons. First, *sli-1* mutations do not suppress the vulval defects of *lin-3, let-60* or *lin-45* mutations to the extent that they suppress *let-23* mutations. Therefore

we consider it unlikely that *sli-1* is interacting with one of these gene products. Second, *sli-1* mutations suppress *let-23* mutations in all of the tissues in which *let-23* is known to function, suggesting that these two genes function in an overlapping set of tissues. Third, mutations at other loci suppressed by *sli-1* (i.e. *lin-2, lin-7,* and *lin-10*) display phenotypes only in the vulva and it has been suggested that these genes function only in this tissue (Ferguson and Horvitz, 1985). If *sli-1* controls the activity of one of these genes, *sli-1* mutations must either allow these genes to function in other tissues to suppress *let-23* mutations or *sli-1* interacts with other gene products in other tissues. Further analysis of this gene will elucidate its role in the negative regulation of EGF receptor.

MATERIALS AND METHODS

Strain Maintenance and Construction

EMS mutagenesis and strain maintenance was as in (Brenner, 1974). X-ray mutagenesis was performed using 1500 rads.

Strain Construction

PS267 [*let-238(mn229) let-23(sy1) + sqt-1(el350)* / + *mnDf67 unc-4(e120)* +] was constructed as follows: *let-238(mn229) unc-4(e120)* / *mnC1[dpy-10(e128) unc-52(e444)]* males were mated to *rol-6(e187) let-23(sy1)* hermaphrodites. Non-Rol cross progeny hermaphrodites were allowed to self-fertilize on individual plates. Animals segregating no Dpy Unc self-progeny (this is the phenotype of *mnC1[dpy-10(e128) unc-52(e444)]* homozygotes) bearing the lethal allele were selected (genotype: + *let-238(mn229)* + *unc-4(e120)* / *rol-*

6(e187) + let-23(sy1) +). From the progeny of these animals, rare Vul non-Rol recombinants (genotype: + *let-238(mn229) let-23(sy1) / rol-6(e187) + let-23(sy1)*) were selected and the *let-238(mn229) let-23(sy1)* chromosome was balanced in *trans* to *mnC1[dpy-10(e128) unc-52(e444)*] by mating rare egg-laying competent non-Rol progeny of the original recombinant to *rol-6(e187) unc-4(e120) / mnC1[dpy-10(e128) unc-52(e444)*] males. This strain (*let-238(mn229) let-23(sy1) /mnC1[dpy-10(e128) unc-52(e444)*]) was heat shocked to obtain male self-progeny. These males were mated to *unc-4(e120) sqt-1(e1350)* hermaphrodites. Cross progeny L4 hermaphrodites (Rol non-Unc) were selected and allowed to self-fertilize on individual plates. From animals which segregate dead larvae but no Dpy Unc-52 self-progeny (thus of genotype: *let-238(mn229) let-23(sy1) ++ / ++ unc-4(e120) sqt-1(e1350)*), rare Dpy non-Unc recombinants (genotype: *let-238(mn229) let-23(sy1) + sqt-1(e1350) / ++ unc-4(e120) sqt-1(e1350)*) were selected and immediately mated to *mnDf67 unc-4(e120) / mnC1[dpy-10(e128) unc-52(e444)*] males. Vul Rol non-Unc cross progeny were selected (genotype: *let-23(mn229) let-23(sy1) + sqt-1(e1350) / + mnDf67 unc-4(e120) +*) and allowed to self-fertilize to establish this reversion strain (**PS267**). Rare non-Rol progeny were discarded throughout the time that this strain was in use.

Animals bearing homozygous *let-23* mutant alleles and carrying the deficiency *meDf3* were constructed as follows:

rol-6(e187) let-23(sy97) / mnCl[dpy-l0(e128) unc-52(e444)] males were mated to hermaphrodites of genotype *meDf3 / unc-l(e538) dpy-3(e27)* hermaphrodites. NonUnc non-Dpy L4 hermaphrodite cross progeny were selected, placed on individual plates, and allowed to self-fertilize. Animals

which segregated dead eggs, rare males (both are indicative of the presence of the deficiency), Rolling (Rol) animals and no Dpy Uncs (the phenotype of both balancer chromosomes) were identified (genotype *rol-6(e187) let-23(sy97) / ++; meDf3 / +).* From these animals, self progeny were scored to determine the number of Rol and non-Rol progeny, the ratio of which was used to determine the ability of this deficiency to dominantly suppress the lethality of this *let-23* allele. The genotype of the Rol animals was confirmed by examining the phenotypes of their self-progeny.

In parallel, wild-type (N2) males were mated to hermaphrodites of genotype *let23(sy97) unc-4(e120); sli-1(sy143).* L4 non-Unc hermaphrodite cross progeny were selected and allowed to self-fertilize on individual plates. All progeny from these animals was scored as Unc or non-Unc to assess the ability of the allele *sli-1(sy143)* to dominantly suppress the lethality of *let-23(sy97).*

let-23(sy1); sli-1(sy143) / meDf3 was constructed by mating males of genotype *let23(sy1) / +; sli-1 (sy143)* to hermaphrodites of genotype *meDf3 / unc-l (e538) dpy3(e27).* Non-Unc non-Dpy L4 hermaphrodite cross progeny were selected, placed on individual plates, and allowed to self-fertilize. Animals which segregated dead eggs, rare males (both are indicative of the presence of the deficiency) and Hin (or Vul) animals but no Dpy Unc animals (therefore of genotype *let-23(sy1) / +; meDf3 / sli-1(sy143)*) were identified. From the progeny of these animals, hermaphrodites displaying vulval abnormalities were selected and allowed to self-fertilize on individual plates (these animals will be of genotype *let-23(sy1); sli-1 (sy143) / meDf3* or

let-23(sy1); sli-1 (sy143)). The genotype of these animals was determined by scoring for the presence of dead eggs and males in their self-progeny.

let-23(sy97) unc-4(e120); sli-1(sy143) / meDf3 animals were constructed by *mating let-23(sy97) unc-4(e120) / mnCl[dpy-l0(e128) unc-52(e444)];sli-1(sy143) males to meDf3 / unc-l(e538) dpy-3(e27)* hermaphrodites. Non-Unc non-Dpy L4 hermaphrodite cross progeny were selected, placed on individual plates, and allowed to self-fertilize. Animals which segregated dead eggs, rare males (both are indicative of the presence of the deficiency), Unc-4 (Unc-4 is distinguishable from both Unc-1 and Unc-52) but no DpyUnc (the phenotype of the balancer chromosomes *mnCl[dpy-l0(e128) unc-52(e444)]* and *unc-l(e538) dpy-3(e27)* were identified (these animals were therefore of genotype *let-23(sy97) unc-4(e120) /++; sli-1 (sy143) / meDf3*). From the progeny of these animals, Unc-4 L4 hermaphrodites were selected and placed on individual plates (these animals are of genotypes *let23(sy97) unc-4(e120); sli-1(sy143) / meDf3* and *let-23(sy97) unc-4(e120); sli-1(sy143)*). Animals bearing the deficiency were identified by the presence of males in their self-progeny. L4 hermaphrodite progeny of these deficiency-bearing animals were individually examined under Nomarski optics for the extent of vulval differentiation, placed on individual plates, and allowed to self-fertilize. Animals which segregated males and dead eggs (and thus were of genotype *let-23(sy97) unc4(e120); sli-1(sy143) / meDf3*)were identified, while animals not bearing the deficiency were used as controls.

let-23(sy97) unc-4(e120); sli-1(sy102) / meDf3 and *let-23(sy97) unc-4(e120); sli-l (sy112) / meDf3* strains were constructed by mating *let-23(sy97) unc-4(e120) / ++; sli-1(sy102* or *sy112)* males to *meDf3 / unc-l(e538) dpy-3(e27)*

hermaphrodites. NonUnc non-Dpy L4 hermaphrodite cross progeny were selected, placed on individual plates, and allowed to self-fertilize. Animals which segregated dead eggs, rare males (both are indicative of the presence of the deficiency) and Unc4s were retained (these animals were therefore of genotype *let-23(sy97) unc-4(e120)/++; sli-l (sy102 or sy112) / meDf3).* Vul Unc-4 progeny of these animals were selected and allowed to self-fertilize on individual plates. From animals which segregated males and dead eggs, L4 hermaphrodite self-progeny were examined individually under Nomarski optics for the extent of vulval differentiation, placed on individual plates, and allowed to self-fertilize. Animals which segregated males and dead eggs (and thus were of genotype *let-23(sy97) unc-4(e120); meDf3 /sli-1 (sy102 or sy112))*were identified, while animals not bearing the deficiency *were* discarded.

let-23(sy97) unc-4(e120); meDf3/+ animals were constructed by mating *let23(sy97) unc-4(e120)/++* males to *meDf3/unc-l(e538) dpy-3(e27)* hermaphrodites. Non-Unc non-Dpy L4 hermaphrodite cross progeny were selected, placed on individual plates, and allowed to self-fertilize. Animals which segregated dead eggs, rare males (both are indicative of the presence of the deficiency) and Vul Unc-4 progeny were retained. From the progeny of these animals, Vul Unc-4 hermaphrodites were selected and allowed to self-fertilize on individual plates (most of these animals are genotypically *let-23(sy97) unc-4(e120); meDf3/+;* others are *let23(sy97) unc-4(e120); +*). Animals bearing the deficiency were identified by the segregation of male self-progeny and dead eggs. L4 hermaphrodite self-progeny of these deficiency bearing animals were examined individually under Nomarski

optics for the extent of vulval differentiation, placed on individual plates, and allowed to self-fertilize. Animals which segregated male self-progeny and dead eggs (and thus were of genotype *let-23(sy97) unc-4(e120); meDf3 / +)* were identified, while animals not bearing the deficiency were discarded.

Other strains were constructed by standard methods.

Mapping

The following double mutant strains were used for mapping:

L.G. I: PS592 *dpy-5(e61); let-23(sy1)*

L.G. II: PS79 *dpy-l0(e128) let-23(sy1)*

L.G. III: PS512 *let-23(sy1); unc-32(e189)*

L.G. IV: PS262 *let-23(sy1); dpy-20(e1282)*

L.G.V: PS593 *1et-23(sy1);dpy-ll(e224)*

L.G. X: PS594 *let-23(sy1); lon-2(e678)*

Subsequent mapping was done with the following strains:

let-23(sy1); unc-3(e151)

let-23(sy1); unc-l (e719) dpy-3(e27)

che-2(e1033)

egl-l 7(e1313)

let-23(sy1); sli-1 (sy143) unc-l (e719)

egl-17(e1313) sli-1(sy143) unc-l(e719)

Mapping crosses were performed as follows: *let-23(sy1); him-5(e1490); sli-l(sy102)* males were mated to hermaphrodites of the mapping strains

above (general genotype of *let-23(sy1); dpy-y).* Cross progeny (non-Dpy-y, genotypically *let23(sy1) /let-23(sy1); him-5(e1490) / +; dpy-y / +; sli-1 (sy102) / +)* hermaphrodites were selected and allowed to self-fertilize. Twelve to twenty-four Suppressed (Hin) nonDpy hermaphrodites were removed to individual plates and allowed to self-fertilize. Linkage was determined based on the fraction of these animals which segregated Dpy progeny. Subsequent mapping was done similarly with animals bearing the X-linked markers *unc-3(e151)* and the double mutant *unc-l(e719) dpy-3(e27).*

Three factor crosses were performed by mating males of genotype *let-23(sy1); him-5(e1490); sup-x* (where *sup-x is* a candidate *sli-1* allele) to hermaphrodites of genotype *let-23(sy1); unc-l(e719)dpy-3(e27).* Cross progeny (non-Unc non-Dpy) L4 hermaphrodites were selected and allowed to self-fertilize. Recombinant (Dpy nonUnc and Unc non-Dpy) hermaphrodites were selected and allowed to self-fertilize. Progeny of these animals were scored for the presence of Hin animals (indicative of homozygous *sli-1).* This cross allows *sli-1* to be distinguished from *suv-l,* a suppressor of *lin-10,* which is to the right of *unc-l* (A. Villeneuve and S. Kim, pers. comm.). All candidate *sli-1* alleles from the **PS267** screen were mapped using this cross. Additional three-factor crosses were performed using animals of genotypes *let-23(sy1); sli-1(sy143) unc-l(e719)* and *egl-17(e1313) sli-1(sy143) unc-l(e719).* These strains were constructed as follows: hermaphrodites of the genotype *let23(sy1)/let-23(sy1); sli-1(sy143) +/+ unc-l(e719)* were allowed to self-fertilize. Unc animals were selected and placed on plates where they were allowed to self-fertilize. Rare HinUnc recombinant animals were selected. By standard crosses, animals of genotype *let-23(+); sli-1(sy143) unc-l(e719)*

were recovered. *egl-17(e1313)* males were mated to these (*sli-1(sy143) unc-l(e719))* hermaphrodites. Cross progeny nonUnc L4 hermaphrodites were selected and allowed to self-fertilize. Egg-laying defective (Egl) animals were selected and allowed to self-fertilize. Rare UncEgl progeny were selected. The *sli-1* genotype of these recombinants was scored after reintroducing the *let-23(sy1)* mutation via a mating.

Complementation tests

Candidate *sli-1* alleles from the *let-23(sy97) unc-4(e120)* reversion screen were tested for complementation of the *sli-1* reference allele *sy102* in the following manner:

Wild type (N2) males were mated to egg-laying competent hermaphrodites of genotype *let-23(sy97) unc-4(e120); sup-x,* where *sup-x* is a candidate *sli-1* allele. Cross progeny males were selected and mated to hermaphrodites of the genotype *unc-54(e190); let-23(sy97) unc-4(e120); sli-1(sy102).* Unc-4 non-Unc-54 L4 hermaphrodite cross progeny were selected, placed on individual plates and subsequently scored for their ability to lay eggs.

Nomarski microscopy

Vulval differentiation. The extent of vulval differentiation was measured as described in (Han and Sternberg, 1990).

P12 vs. P11. L3 and L4 animals were scored for the apparent transformation of P12.pa to P11.p, but hypothetically the transformation is

actually P12 to P11, based on a more detailed analysis of the mutations in the pathway (Fixsen et al., 1985).

Male tail abnormalities. Male tail phenotypes were scored by Helen Chamberlin by examining young adult males for the "crumpled spicule" phenotype associated with several *let-23* mutations (Aroian and Sternberg, 1991).

Ablation

Ablation of the gonad (including anchor cell) precursor cells was performed as described by (Avery and Horvitz, 1987, Sulston and White, 1980).

Other measurements of suppression of lethality

lin-3. The strain + *lin-3(n378)* + *unc-22(e66)* / *unc-24(e138) lin-3(n1059) dpy20(e1282)* +; *sli-1(sy143)* was constructed by standard methods. No Dpy Uncs were found in several generations of maintaining this strain. Thus, the lethality associated with the allele *lin-3(n1069) is* not suppressed.

let-23. Let-23(mn224 or *mn23) unc-4(e120) / mnC1[dpy-10(e128) unc-52(e444)*]males were mated to *sli-1(sy143)* hermaphrodites. Possible cross progeny L4 hermaphrodites were selected, placed on individual plates, and allowed to self-fertilize. Several animals bearing the *let-23 unc-4* chromosome were found, but no non-recombinant Unc-4 animals were found in their progeny. A similar cross was performed with similar results using *rol-6(e187) let-23(sy15) / mnC1[dpy-10(e128) unc-52(e444)]* males.

let-60. Using standard methods, a strain of genotype + *let-60(n1046gf)* + *unc22(s7)/unc-24(e138) let-60(sy100dn) dpy-20(e1282)* +; *sli-1(sy102)* was constructed. Approximately 100 Unc-24 Dpy animals were selected and placed on a single plate. None of these animals produced any viable progeny. Thus *let-60(sy100dn); sli-1(sy102)* homozygotes are inviable. Suppression of the lethality of the subviable reduction of function allele *let-60(n2021)* was measured by allowing egg-laying competent hermaphrodites of genotype *let-60(n2021); sli-1(sy143)* to lay a cohort of eggs on a plate. The hermaphrodites were removed, the eggs counted, and two days later the number of viable animals was determined.

lin-45. Rare non-Egl animals of genotype *lin-45(sy96) unc-24(e138); sli-1(sy143)* were allowed to lay a cohort of eggs on a plate. These eggs were counted. Two days later, viable animals were counted.

RESULTS

I. Isolation of suppressors of the *let-23* vulval defect

We screened for suppressors of weak (viable) alleles of *let-23,* an EGF-R homolog to identify genes that interact with *let-23.* We used two *let-23* genotypes as starting strains (Fig. 2a. 2b); one of these strains, **PS267**, balances a weak *let-23* allele in *trans* to a deficiency, the other is homozygous for a more severe allele. From these screens we recovered partially overlapping sets of suppressor mutations (Fig. 3).

The first screen takes advantage of the weak allele *sy1* . *sy1* is a tissue-specific allele in that animals homozygous for this allele display abnormal

vulvae but are apparently wild-type in all other tissues that require *let-23* ~Aroian, 1991#4723. The strain **PS267** balances this allele in *trans* to a deficiency of the locus (Fig. 2a). Animals of this genotype almost never lay eggs (0/3600 in contrast to 7% of *sy1* homozygotes, (Aroian and Sternberg, 1991)) but are otherwise wild-type. Approximately 75,000 EMS (ethyl methane sulfonate) mutagenized F1 chromosome sets were screened for animals competent to lay eggs. Mutations at a number of loci were recovered in this screen (Fig. 3). A single *let-60 ras(gf)* allele was recovered. This allele, like the other four *let-60(gf)* alleles, is semi-dominant (data not shown). This allele was recovered as an F1 (dominant) suppressor of *let-23* and was mapped to the *let-60* region of linkage group IV (data not shown). Subsequently, this allele was sequenced and shown to be identical to the other *let-60 ras(gf)* alleles (Beitel et al., 1990). Two new *unc-101* alleles were also recovered. The genetics of the *unc101* locus and its role in vulval development will be described elsewhere. Several alleles at a new locus, *sli-1* (suppressor of lineage defect) were recovered. These mutant alleles all act as silent suppressors of mutations at *let-23*. Generally, the vulval phenotype of animals of genotype *let-23; sli-1* was either wild-type of Hin (Fig. 4, Table 5). The allele *sy102* was chosen for further analysis. *sy102* causes a slightly more penetrant suppression of the *let-23(sy1)* vulval defect than the other alleles recovered in this screen. A total of 17 alleles were recovered in this screen, at least seven of which are *sli-1* alleles (*sy102, sy104, sy106, sy107, sy112, sy114, sy115, sy162,* and *sy185*). Several alleles; were recovered which are not members of the three loci described. However, we have not

characterized these alleles, except to determine that they are not intragenic *let-23* revertants, *sli-1, unc-101,* or *let-60* alleles.

PS267 was also mutagenized with 1500 R of X-rays in a screen for X-ray induced suppressors. Approximately 36,000 mutagenized F1 chromosome sets were screened for F1 (dominant) or F2 (recessive) suppressors and an additional 650,000 mutagenized F1 chromosome sets were screened for F1 (dominant) suppressors only. However, no true-breeding suppressors were recovered from these screens.

A more severe allele of *let-23, sy97,* was used as the parent stock for the second screen. Animals homozygous for this allele are subviable (~85% die). Viable animals are Vul and display very little vulval differentiation. Approximately 14,000 mutagenized F1 chromosome sets were screened for suppressors. At least 26 alleles were recovered. One of these, *sy97sy122,* is a dominant suppressor of *let-23* that is tightly linked to *let-23.* This allele has been shown to be an intragenic revertant of *let-23* (Aroian et al., 1993). Most of the other alleles fail to complement *sy102,* a *sli-1* reference allele. This is not conclusive proof that these alleles are *sli-1* alleles, since at least some *sli-1* alleles are semi-dominant suppressors of *let-23(sy97)* (Table 1). Two of these alleles, *sy143* and *sy129* were chosen for further analysis. These alleles were strong suppressors of the Vul phenotype of this *let-23* allele and were strong suppressors of *let-23(sy97)* in *trans* to *sy102.*

II Mapping and Complementation

sli-1 was mapped to the left arm of the X chromosome. Three factor crosses place *sli-1* in the interval between *unc-l* and *egl-17*(Fig. 4). Specifically, five of twelve Unc non- Egl recombinant chromosomes from an

egl-17(e1313) sli-1(sy143) unc-l(e719)/+++ heterozygous hermaphrodite carried the *sli-1* mutation, placing *sli-1* in the interval between *egl-17* and *unc-l.* Deficiencies that delete flanking markers as well as *sli-1* uncover the suppression phenotype of the locus (Table 1). Duplications that include flanking loci complement the suppression phenotype (Table 1). All of the alleles isolated in the *let-23(sy97)* reversion screen were tested for complementation against the reference allele *sy102* and most failed to complement this allele. All of the *sli-1* alleles isolated as **PS267** suppressors also fail to complement the allele *sy102* except for *sy112* (Table 1). *sy112* fails to complement other mutations at this locus and maps to the left of *unc-l* . *sy112 is* thus considered to be an allele of *sli-1.*

III. *sli-1 is* a silent locus

All of the *sli-1* alleles that we have analyzed display no obvious phenotype in the absence of another mutation (Table 2) except for a low penetrance notchhead phenotype (Fig. 5). This phenotype is present but rare in all *sli-1* genotypes examined to date.

sli-1 mutations may act as a synthetic multivulva mutation, such that animals bearing so called "class A" mutations in combination with a strong *sli-1* allele display a temperature sensitive Muv phenotype. Specifically, animals of genotype *sli-1(sy143) unc-l (e719) lin-l5(n433), sli-1(sy143) unc-l(e719) 1in-l5A(sy197),* or *lin-8(nlll); sli-1(sy143)* display no mutant phenotype at 20° but are Muv at 25°. The vulval differentiation seen in these animals is independent of the inductive signal, as ablation of the gonad in L1 animals did not disrupt the ability to form vulval tissue (data

not shown). *sli-1(sy129)* also interacted with class A mutation (data not shown). However, animals bearing the weaker alleles *sy102* or *sy112* in combination with these *lin-8* or *lin-15(A)* alleles are not multivulva. This might be a somewhat non-specific effect, since the double mutant combination *let-60(n1046gf)*; *sup-7(st5)* acts as a class A synthetic multivulva mutation, despite the fact that neither *let-60* or *sup-7* has been otherwise implicated in the syn-muv mediated negative regulation pathway.

IV. *sli-1* mutations suppress vulval defects of many, but not all, mutations of genes required for vulval development

To determine whether suppression by *sli-1* was limited to *let-23* or was general to mutations at other loci required for vulval induction, we examined the ability of *sli-1* to suppress mutant phenotypes associated with mutations in other genes in the vulval induction pathway. This double mutant analysis of *sli-1* suggests that *sli-1* acts as a negative regulator of either *let-23* or another gene acting at about the same step in the vulval development pathway.

The Vul phenotype of some mutations at an early acting gene, *lin-3* is suppressed, while other mutations at the *lin-3* locus are not suppressed by *sli-1* mutations. Animals bearing a mutation at both *lin-3* and *sli-1* are frequently as defective in vulval development as are *lin-3; +* animals. However, animals bearing certain *lin-3; sli-1* allelic combinations display a less penetrant vulval defect than do control animals not mutant for *sli-1*. A strong *lin-3* genotype (a reduction of function allele in *trans* to a putative

null allele) is suppressed from an average of 0.1 to 0.6 VPCs forming vulval tissue per animal (Table 2, Hill and Sternberg, pers. comm.). Thus *sli-1* partially suppresses the vulval defect of a severe *lin-3* allelic combination. This reflects residual or suppressible *lin-3* activity, since vulval differentiation in *sli-1* animals requires the presence of the gonad (Table 5). Homozygous *lin-3(n1059)* progeny of this *trans* heterozygote are inviable, indicating that this *sli-1* allele is not sufficient to suppress a *lin-3* null. Suppression of *lin-3* does not result in Hyperinduced animals, rather, there is an increase in the number of VPCs forming vulval tissue to a maximum of three. This may reflect the inability of *sli-1* to cause a *lin-3* mutant strain to display the Hin phenotype, alternatively, it may simply reflect the overall low level of induction in these doubly mutant strains. The Hin phenotype has not been observed in animals bearing a *lin-3* reduction-of-function mutation. It seems unlikely that *lin-3* is the target of *sli-1* regulation. A suppressor of *lin-3* mutations would not be expected to suppress mutations in genes acting after *lin-3,* such as *let-23.* It is possible that a *lin-3* suppressor mutation might be sufficient to suppress reduction of function mutations at later steps, however, this should be constant (reduction of function mutations of all genes that act after *lin-3* should then be suppressed) and this does not hold true for *sli-1,* since mutations at *let-23* are suppressed while mutations at *let-60* and *lin-45* are not suppressed.

All viable *let-23* mutations examined are suppressed. In most cases, animals of genotype *let-23; sli-1* are likely to display the Hin phenotype. Animals bearing the *let-23* alleles *sy1, sy12,* or *nl045* in combination with a *sli-1* mutation are frequently Hin (Table 3). Animals bearing certain weak

sli-1 alleles (such as *sy115* or *sy263*) in combination with *let-23(sy1)* display less than wild-type vulval differentiation (Table 1). Most animals of these genotypes are Vul; however, wild-type and Hin animals are not uncommon. Suppression to wild-type is uncommon; only animals bearing the *let-23* allele *sy97* and a subset of *sli-1* mutations (the alleles *sy129,* and *sy143* but not *sy102* or *sy112)* approach wild-type (Table 1). Most animals of these genotypes are wild-type; a small percentage of animals display a Vul phenotype. Hin animals are not seen in any animal bearing the *let-23* allele *sy97,* regardless of the *sli-1* genotype. The weak allele *let-23(sy1) is* suppressed by all of the *sli-1* alleles tested. These *let-23(sy1); sli-1* animals frequently display the Hin phenotype and only rarely do animals display less than wild-type vulval differentiation (Table 1). The Hin allele *let-23(n1045) is* suppressed 15° but not at 25° by the *sli-1* allele *sy143.* Animals of this genotype *(let-23(n1045); sli-1 (sy143))* are no longer temperature sensitive (animals of the genotype *let-23(n1045)* display less than wild-type vulval differentiation at 15°; at 25°, these animals display greater than wild-type vulval differentiation, Table 3). In contrast, *let-23(n1045); sli-1(sy143)* double mutant animals (at any temperatures) resemble *let-23(n1045)* animals at 25° (Table 3). *let-23(sy12),* an allele similar in severity to *let-23(sy97),* is also suppressed to an apparent Hin phenotype by the *sli-1* allele *sy143* (Table 3). The Hyperinduced phenotype then seems to be more dependent on the *let-23* genotype than the *sli-1* genotype (i.e., mutations which suppress *let-23(sy97)* to wild-type or nearly wild-type are sufficient to cause the Hin phenotype in combination with other *let-23* alleles, but no *sli-1* alleles are apparently sufficient to cause the Hin phenotype in an animal

of genotype *let- 23(sy97); sli-1*). We therefore suggest that this reflects the fact that the allele *let-23(sy97)* is able to activate a second negative regulation pathway (not involving *sli-1*) that functions to refine the pattern of induced cells, while other mutations at *let-23* are unable to do so.

Iin-2, lin-7, and *lin-10* mutations are suppressed. All allelic combinations result in frequent Hin animals (Table 2). This is true of animals homozygous for putative null alleles of these loci in combination with a *sli-1* allele. These are the only genes required for vulval differentiation of which putative null alleles are suppressed by *sli-1* mutations. It is unclear whether this is simply because animals bearing these null alleles are viable (and the Vul phenotype of animals homozygous for null alleles of other genes would be suppressed if these animals were viable) or whether it reflects the ability of *sli-1* mutations to specifically suppress putative null alleles of the *lin-2, lin-7,* and *lin-10* loci. Animals homozygous for *lin-2, lin-7,* or *lin-10* null alleles still display significant residual vulval differentiation (Table 2), in contrast to what is believed to be the case for null alleles of *lin-3, let-23,* or *let-60.* It is possible that *sli-1* suppresses these null alleles indirectly, by increasing whatever activity is responsible for residual differentiation in the absence of *lin-2, lin-7* or *lin-10* gene products. A *sli-1* mutation increases the penetrance of the Hin phenotype of animals which are homozygous for either *lin2(n768)* or *lin-7(n308).* Animals homozygous for these alleles frequently display the Hin phenotype in the absence of a *sli-1* mutation (Jongeward and Sternberg, in prep.). These double mutant animals are very similar in phenotype to those

of double mutant animals bearing putative null alleles at these loci and *sli-1* mutations.

The vulval differentiation defect associated with a weak mutation at *sem-5* is suppressed to a nearly wild-type phenotype by *sli-1(sy143)* (Table 2). Phenotypically, these *sli-1 sem-5* animals are similar to *let-23(sy97); sli-1* animals, in that no Hin animals are observed. A *sli-1* mutation does not suppress the sex myoblast phenotype associated with a *sem-5* mutation. The lethality associated with this *sem-5* mutation is partially suppressed. The brood size of *sem-5;* + animals is approximately 21. In contrast, the brood size of *sli-1 sem-5* animals averages 44. This may reflect a slight ability on the part of the *sli-1 sem-5* animals to lay eggs. However, it seems unlikely that these animals lay enough eggs to double the number of progeny generated.

let-60 mutations are apparently not suppressed. Animals bearing a homozygous *let-60(dn)* allele and the *sli-1* allele *sy102* display no vulval differentiation (Table 2). This may be misleading, as *sli-1 is* insufficient to suppress true nulls of *let-23* or *lin3,* and *let-60(dn)* homozygotes are likely to be more similar to true nulls than they are to hypomorphs. *let-60(dn)* homozygotes segregate only dead larvae. This phenotype is not suppressed by *sli-1(sy102).* A very weak hypomorphic allele of *let-60, n2021*, may be slightly suppressed by a *sli-1* mutation. *let-60(n2021)* animals display a slight defect in vulval differentiation (an average of 2.5 VPCs undergo vulval differentiation, Table 2). This defect is lessened by a *sli-1* mutation, such that an average of three cells undergo vulval differentiation in animals of genotype *let-60(n2021); sli-1(sy143)* (Table 2). This is not

completely wild-type, as animals are observed with less than three cells assuming vulval fates. As the *let-60(n2021)* allele is quite weak, it is difficult to interpret the meaning of the phenotype of this double mutant.

lin-45 is also not suppressed by *sli-1* . The extent of vulval induction in *lin45(sy96); sli-1(sy143)* is similar to the amount of induction seen in *lin45(sy96);* + animals (Table 2). In addition, the lethality associated with this allele is not suppressed. Specifically, like animals of genotype *lin-45(sy96); sli-1 (+),* very few animals of the genotype *lin-45(sy96); sli-1(sy143)* are viable, in marked contrast to animals bearing a similar subviable allele of *let-23* and *sli-1(sy143). let-23(sy97) is* suppressed to complete viability by *sli-1(sy143),* whereas there is no apparent suppression of the lethality of the *lin-45(sy96)* allele.

Thus, mutations at *sli-1* are sufficient to suppress the vulval defect of some *lin-3* mutations, suppress the vulval defects of weak *let-23* mutations, suppress the vulval defects of all *lin-2~ lin-7,* and *lin-10* mutations, but do not suppress the vulval defects associated with mutations at *let-60* or *lin-45.* We conclude that *sli-1* acts as a negative regulator of the vulval induction pathway at a step prior to the activities of *let-60 ras* and *lin-46 raf* (Fig. 5).

V. Other Tissues

let-23 functions in several developmental decisions in a number of tissues. To determine if *sli-1* mutations are specific negative regulators of vulval induction or if these mutations function as negative regulators in other tissues as well, we examined the ability of *sli-1* mutations to suppress defects associated with *let-23* mutations in other tissues. We therefore

examined the phenotypes of a number of allelic combinations of several hypomorphic *let-23* alleles and the strong *sli-1* allele *sy143*. *sli-1* suppresses all of the identified *let-23* defects associated with weak alleles of *let-23* (Table 4). This includes the lethality associated with some of the weak *let-23* mutations. For instance, the lethality of *sy97 is* suppressed semidominantly by the allele *sli-1 (sy143)* and dominantly by the deficiency of the region *meDf3* (Table 4). Similarly, the subviability of the *let-23* allele *nl046 is* also suppressed by *sli-1 (sy1 43)*. However, neither true null alleles such as *mn23* and *sy15* nor the near-null allele *mn224* are measurably suppressed (Table 4). Mutations at the *sli-1* locus are apparently not sufficient to bypass the requirement for *let-23* for viability. This is consistent with the gonad dependence of *let-23; sli-1* doubly mutant animals (Table 5). If a *sli-1* mutation bypassed the requirement for *let-23* in the vulva, then vulval differentiation would presumably not require the inductive signal. Instead, *sli-1* mutations seem to modify the function of *let-23*.

The sterility of the allele *let-23(sy12) is* also suppressed. Most animals of the genotype *let-23(sy12); sli-1 (+)* are inviable, while escapers are sterile Vuls. In contrast, animals of the genotype *let-23(sy12); sli-1(sy143)* are somewhat viable, Hin (Table 3), and about 60% of these animals are fertile (Table 4).

let-23 is also required for development of the male copulatory spicule. Males homozygous for the allele *sy97* generally display spicule defects, whereas significant numbers of animals of the genotype *let-23(sy97); sli-1 (sy143)* /Ø have wild-type spicules (Table 4).

Some *sli-1* mutations suppress the transformation of the P12 cell to P11 in *let-23(sy97)* animals, although both the transformation by *let-23(sy97)* and the suppression by *sli-1* are incomplete. This suppression varies, as certain *sli-1* alleles suppress this phenotype, while other *sli-1* alleles do not noticeably suppress this phenotype (data not shown).

The lethal phenotypes of mutations at *lin-3, let-60,* and *lin-45* are not suppressed by mutations at *sIi-1.* This is true for the *lin-3* null allele *nl059,* the *let-60* dominant negative *sy100,* and the *lin-45* hypomorph *sy96* (data not shown).

VI. Null Phenotype

To characterize the *sIi-1* locus genetically, we attempted to recover null alleles of the locus. A deficiency of the locus is viable in *trans* to the allele *sli-1(sy143)* in both a wild-type background and in a *let-23(sy1)* background. Animals of the genotype *let-23(sy1); sli-1(sy143) / meDf3* display a phenotype similar to that of *let23(sy1); sli-1(sy143),* in that most animals of either genotype are competent to lay eggs and have prominent pseudovulvae. Therefore it was possible to recover null alleles in *trans* to this *sli-1* allele. One *sli-1* allele was recovered in a screen of 5,200 mutagenized haploid genomes. This allele, *sy263*, is a weaker suppressor of *let23(sy1)* than the reference alleles, but causes similar phenotypes. Specifically, animals of genotype *let-23(sy1); sli-1(sy263)* display 82% vulval differentiation. Analysis of deficiencies of the region suggest that the phenotypes of the stronger alleles are indeed strong reduction of function phenotypes of the locus. As mentioned above, the *trans* heterozygote *(let-23(sy1); meDf3 / sli-1(sy143)) is*

suppressed for the *let-23* vulval defect. These animals are similar to *let-23(sy97); sli-1(sy143)* double homozygotes. Other, weaker *sli-1* alleles are enhanced by *meDf3* (Table 1). Thus, the allele *sy143* is about as severe as *meDf3* and the alleles *sy102* and *sy112* are weaker. The allele *sy143* behaves similarly to *meDf3* when measured in *trans* to *sli-1(sy102)* in a *let-23(sy97)* background (Table 1), suggesting that *sli-1(sy143)* is nearly as severe as the deficiency. This comparison holds true for heterozygous animals. *let-23(sy97); meDf3* / + heterozygotes are occasionally egg-laying competent and display *43%* vulval differentiation. *let-23(sy97); sli-1(sy143)* / + display less vulval differentiation than *Df* / + animals but more than + / + animals (Table 1). Thus, *meDf3 is* more severe than *sy143.* Similarly, the allele *sy143* is a strong suppressor of *let-23(sy97)* lethality. Approximately 80% of animals of genotype *let-23(sy97); meDf3* /+ or *let23(sy97); sli-1(sy143)* /+ are viable, whereas ~15% of animals of the genotype *let23(sy97); sli-1(+)* are viable (Table 4), suggesting that loss of one copy of the locus is sufficient to suppress the lethality of weak alleles of *let-23.* Thus, *meDf3* and *sli-1(sy143)* are equal in their ability to suppress this phenotype. We conclude that the alleles which we have characterized are strong reduction of function alleles at the *sli-1* locus. We base this on five criteria. First, the allele *sy143 is* very similar to the deficiency *meDf3* in all examined genotypes, although *sy143 is* weaker than the deficiency in some phenotypes. Second, *sy143 is* the most severe allele in an allelic series defined by the alleles recovered as suppressors of *let-23(sy97),* but is not exceptional when compared to the other alleles recovered. Third, in a non- complementation screen, we recovered an allele similar to the previously identified alleles. Fourth, the

screen for suppressors of *let-23(sy97)* should allow the recovery of *sli-1* null alleles as dominant suppressors of *let-23* at a reasonable frequency, since a null allele at this locus should suppress the lethality of this *let-23* allele and should semi-dominantly suppress the vulval defect of this allele. We recovered a large number of *sli-1* alleles in this screen, all of which are homozygous viable, at least some of which are dominant suppressors of the *let-23(sy97)* Vul phenotype. If the null phenotype of *sli-1* were lethal or sterile, such alleles would have been recovered in this screen as weak dominant suppressors of the vulval defect which were recessive lethals. Fifth, we recovered alleles of *sli-1* as suppressors of PS267 at a frequency consistent with the recovery of null alleles (1/10,000).

VII. Dosage Sensitivity

The *sli-1* locus shows dosage sensitivity for suppression of the *let-23* in at least two tissues. For instance, a higher percentage of animals of genotype *let-23(sy97) / let-23(sy97); sli-1(sy143) /* + animals are viable than are *let-23(sy97) / let- 23(sy97);* + / + control animals (Table 4). This is also true of *let-23(sy97) / let -23(sy97); meDf3 /* + animals (Table 4). These *let-23(sy97); meDf3 /* + animals also display suppression of the vulval defect (Table 1). Animals of genotype *let-23(sy1) / let-23(sy1); mnDp68f[sli-1(+)] sli-1(sy143) / sli-1(sy143)* or *let-23(sy1) / let- 23(sy1); mnDp68f[sli-1(+)] sli-1 (+) / sli-1 (+)* display a more penetrant vulval defect than do animals of genotype *let-23(sy1) / let-23(sy1); 23(sy1) / let-23(sy1); sli-1 (sy143) / sli-1 (+)* (Table 2). *let-23(sy1)* animals bearing *sli-1*(+) transgenes are also enhanced for the vulval defect of *let-23* (C. Yoon, G. Jongeward, and P. Sternberg, unpub. obs.). Thus, this locus is haplo-insufficient and displays an opposite phenotype in a triplo-sli-1(+) genotype *(sy143 / sy143 = sy143 / Df > Df* + > +/+ > +/*sy143 / sy143* > +/+/+).

DISCUSSION

We have recovered and characterized a number of suppressors of *let-23,* an EGF-receptor homolog required for proper *C. elegans* vulval differentiation. These suppressors were recovered in two screens. These two screens impose different selective constraints. Reversion of **PS267** (a very weak *let-23* allele balanced in*trans* to a deficiency of the locus) selected solely for suppression of a severe *let-23* Vul phenotype. In contrast,

reversion of *let-23(sy97) unc-4(e120)* selected strongly for dominant suppression of lethality and suppression of Vul. Not surprisingly, the alleles recovered as suppressors of the vulval defect of *let-23(sy97)* were also dominant suppressors of the lethality of this allele. Of the four complementation groups which we have characterized, mutations were common at one locus *(sli-1)* and rare at the other three. The rare class included mutations at two loci which were expected to function as suppressors of *let-23,* these being an intragenic revertant and a *let-60 ras (gf)* mutation. The third rare locus, *unc-101,* will be described elsewhere (Lee, Jongeward, and Sternberg, in prep.).

sli-1 alleles were recovered frequently in both strains. The reversion of **PS267** yielded several alleles at this locus at a frequency consistent with the recovery of null alleles (1/10,000). Alleles at this locus were recovered very frequently as suppressors of *let-23(sy97)* (approximately 1/500). This high frequency reflects the ability of these *sli-1* mutations to dominantly suppress the lethality of the *let23(sy97)* allele. Since over 80% of animals homozygous for the *let-23(sy97)* allele die, any mutation which is capable to suppress this lethality dominantly will be preferentially represented in the F2 (any F1 animal bearing a putative dominant suppressor of lethality will be viable, whereas most animals not bearing such a mutation will likely die). Thus, this screen is five to tenfold more extensive for dominant suppressors of lethality provided that these mutations are also capable to suppress the vulval defect. Thus, this screen allows a very powerful selection for *sli-1* alleles and intragenic *let-23* revertants.

We do not believe that *sli-1 is* an informational suppressor. Two classes of informational-suppressor mutations have been characterized in *C. elegans*. *sli-1 is* unlike either of these. *sli-1 is* clearly not an amber suppressor, as it suppresses several alleles which are not amber suppressible, based on characterization of the molecular lesions associated with these mutations, as well as failing to suppress several mutations which are suppressed by amber suppressors (such as *let-60(n1046)* and *unc-24(e138))*. A second class of informational suppressor is the *smg* class of genes (Hodgkin et al., 1989). *sli-1 is* unlike these genes, as it does not interact with *dpy-5* in a manner consistent with these mutations, nor does it display the male abnormal phenotypes associated with these mutations(Hodgkin et al., 1989). *sli-1* does not suppress only mutations at 3' splice acceptor sites. Two of the mutations of this type mutations are suppressed *(let-23(sy97)* and *let-23(n1045)* while two others are (*lin-45(sy96)* and *dpy-10(e128)*. If *sli-1* is an informational suppressor, it is of a novel class.

Mutations at the *sli-1* locus are capable of suppressing a subset of mutations of several of the genes *(lin-3, let-23, lin-2, lin-7,* and *lin-10)* required for the formation of vulval tissue. *sli-1* mutations are insufficient to suppress null alleles of *let-23* or *lin-3*. Thus, a *sli-1* mutation does not bypass the requirement for these products, suggesting that this gene acts as a negative regulator of the process of vulval induction, rather than as an antagonist acting directly within the pathway. Mutations at the *sli-1* locus suppress putative null alleles of *lin-2, lin-7,* and *lin-10*. If these alleles are indeed null (Ferguson and Horvitz, 1985, Horvitz and Sulston, 1980), then

the loci are not entirely essential for vulval induction, since animals homozygous for putative null alleles of any of these loci display significant amounts of residual vulval differentiation (Ferguson et al., 1987, Sternberg and Horvitz, 1989, Sulston and Horvitz, 1981). Suppression of these null alleles may therefore take place as a result of either 1) suppression of the null alleles directly or 2) increased activation of a "bypass pathway" which is normally responsible for the residual induction seen in these "null" animals. Animals homozygous for mutations at the *sli-1* locus as well as a mutation at *let-23, lin-2, lin-7, or lin-10* frequently form excessive vulval tissue in response to the inductive signal. One allele of *let-23, sy97,* does not display this excessive differentiation when combined with a *sli-1* mutation. We believe that this reflects an inherent ability of the *sy97* allele to activate a *(sli-1* independent) pathway of negative regulation that normally functions to refine the pattern of induced cells, perhaps by activating lateral inhibition among cells that receive inductive signal. Other alleles (such as *sy1, nl045,* and *sy12)* are unable to activate this regulation. Normally, this defect would be masked by the failure in signal transduction (since the inability to down regulate would be irrelevant to a cell which cannot respond to the inductive signal). This defect in signal transduction is suppressed by the *sli-1* mutation. Mutations at two of the later acting genes in the pathway, *let-60 ras* and *lin-45 raf,* are not suppressed by *sli-1* mutations. *sli-1* suppresses neither the vulval defects nor the lethality of these mutations. We imagine that *sli-1* acts as a negative regulator, acting before the activity of either *let-60 ras* or *lin-45 raf* (Fig. 7).

We do not believe that the interaction certain *lin-15* class A alleles necessarily reflects a function for *sli-1* in the pathway that controls *lin-15*. The *let-60 ras(gf)* alleles when in a strain also bearing mutations in *lin-9* and in the amber suppressor *sup-7* display a synthetic Muv phenotype. This *let-60* mutation is not a true amber stop mutation, instead, this double mutant combination seems to sensitize the VPCs to the effect of a mutation in a class B gene. This interaction with *lin-l5* is unlike that seen in animals of genotype *let-23(sy1); sli-1 (sy143),* as the vulval differentiation in the *sli-1 (sy143) lin-15(n433)* strain is signal independent, whereas the differentiation in the *let-23(sy1); sli-1 (sy143)* strain is signal dependent.

The *sli-1* locus displays dosage sensitivity. Animals bearing duplications of the *sli-1* region in a weak *let-23* background display an enhanced Vul phenotype regardless of the chromosomal genotype at the *sli-1* locus. Thus, three copies of *sli-1* enhance *let-23*. Deficiencies of the *sli-1* region or strong *sli-1* mutations in *trans to* a wild-type copy of the *sli-1* locus result in strong suppression of the lethality of a subviable *let-23* allele (this is also true of animals heterozygous for a strong *sli-1* allele), as well as partial suppression of the vulval defect of this allele. Thus, the locus is haplo-insufficient. We interpret this to be dosage sensitivity of the *sli-1* locus by three criteria: 1) the chromosomal rearrangements are relatively small and do not include the nearest locus known to function as a negative regulator of the process of vulval induction, *suv-l* (S. Kim and A. Villeneuve, pers. comm.) and it seems unlikely that there is a third locus in this small region which is involved in negative regulation of vulval induction, 2) most *sli-1* alleles display a semidominant phenotype in the

suppression of the lethality of *let-23(sy97),* indicating that lesions affecting this gene only are dosage sensitive and 3) extrachromosomal arrays that rescue the *sli-1* mutant phenotype also enhance the severity of the *let-23* defect (C. Yoon, G. Jongeward, and P. Sternberg, unpub. obs.). We rationalize the incongruous result of the *Dp/sy143/sy143* strain (enhancement of *let-23(sy1)* equivalent to that seen in the *Dp/+/+* strain) as the result of the use of a non-null *sli-1* allele.

It is not clear if the *sli-1* gene product directly interacts with the vulval induction pathway. If there is a direct interaction, the best candidate for this interaction would be *let-23,* as 1) *let-23 is* suppressed strongly, and 2) mutations at *sli-1* are competent to suppress *let-23* mutations in several tissues. It is difficult to postulate a simple model for *sli-1* interacting with *lin-10,* for instance, since this gene is required neither for viability, fertility, nor male tail development, all of which are tissues in which *sli-1* functions. It is possible that *sli-1* interacts with different genes in different tissues to control the functions of this signal transduction pathway, for instance, interacting with *lin-10* in the vulva, and interacting with other (unidentified) tissue specific genes in other tissues. However, the simplest model is that *sli-1 is* a negative regulator of *let-23.* Direct negative regulators of *let-23* might include gene products involved in the control of Let-23 recycling (i.e., genes required for proper delivery of activated receptor to coated pits and subsequently to the lysosome for degradation), specific tyrosine phosphatases that antagonize the putative Let-23 tyrosine kinase activity, or repressors of transcription of genes required for signal transduction. Indirect negative regulators might include proteases that

degrade intercellular proteins or Let-23, components of the basement membrane that normally function to slow the diffusion of the inductive signal, gene products that are required for the fusion of uninduced VPCs to hyp7, and many other products.

Screens such as those described in this report will be useful in identifying new loci which are silent in wild-type backgrounds. *sli-1* would never have been identified in a standard screen for mutations. The only phenotype associated with a *sli-1* mutation in a wild-type background is a low penetrance "notchhead." Current estimates of gene number based on genSomic sequencing are much higher than those based on genetic screens. Like *sli-1,* some of these uncharacterized loci are likely redundant or modifiers of other genes. Such loci will only be identified as enhancers or suppressors of specific phenotypes.

Figure legends

Figure 1.

Schematic of vulval induction showing representative phenotypes of a number of types of mutations in the presence and absence of an anchor cell.

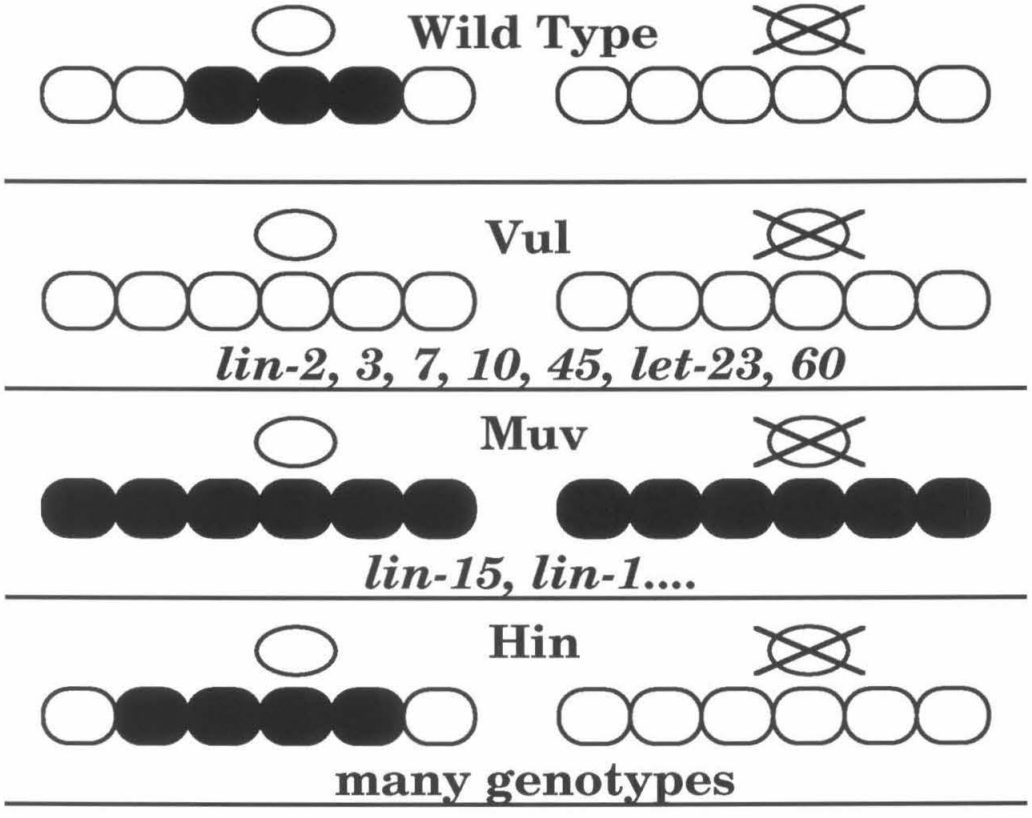
Wild Type
Vul
lin-2, 3, 7, 10, 45, let-23, 60
Muv
lin-15, lin-1....
Hin
many genotypes

Figure 1

Figure 2. Screens for mutations described in this study.

2a. Reversion of **PS267**. The *sqt-1* allele used is semi-dominant, thus all genotypes can be scored for this marker and many recombinant chromosomes can be discarded.

2b. Reversion of *let-23(sy97)*. 85% of animals homozygous for this allele die as L1 larvae. This screen exerts a very powerful selection for dominant suppressors of this phenotype.

2c. Non-complementation screen to recover new alleles of *sli-1*.

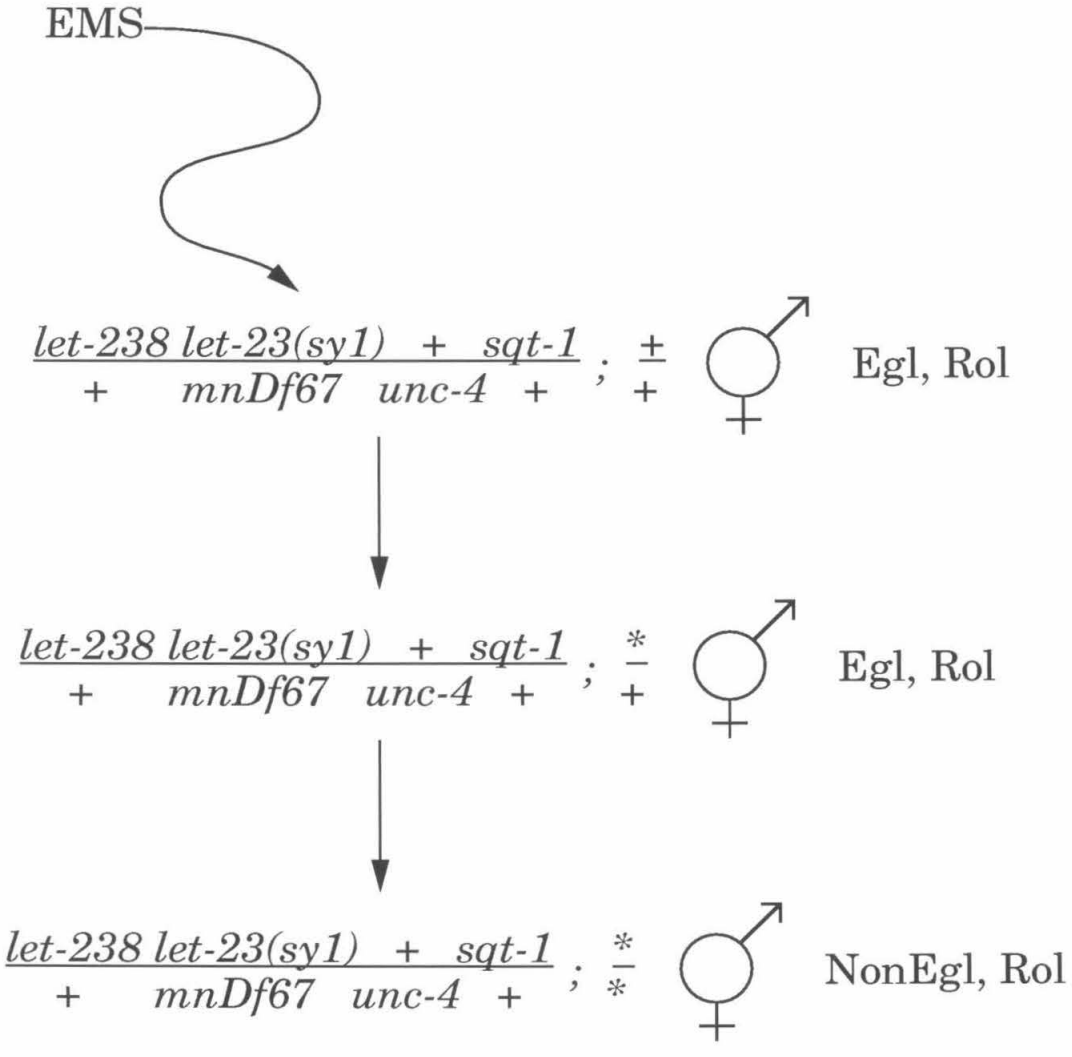
EMS
let-238 let-23(sy1) + sqt-1 / + mnDf67 unc-4 + ; ± / +
Egl, Rol
let-238 let-23(sy1) + sqt-1 / + mnDf67 unc-4 + ; * / +
Egl, Rol
let-238 let-23(sy1) + sqt-1 / + mnDf67 unc-4 + ; * / *
NonEgl, Rol

Figure 2a.

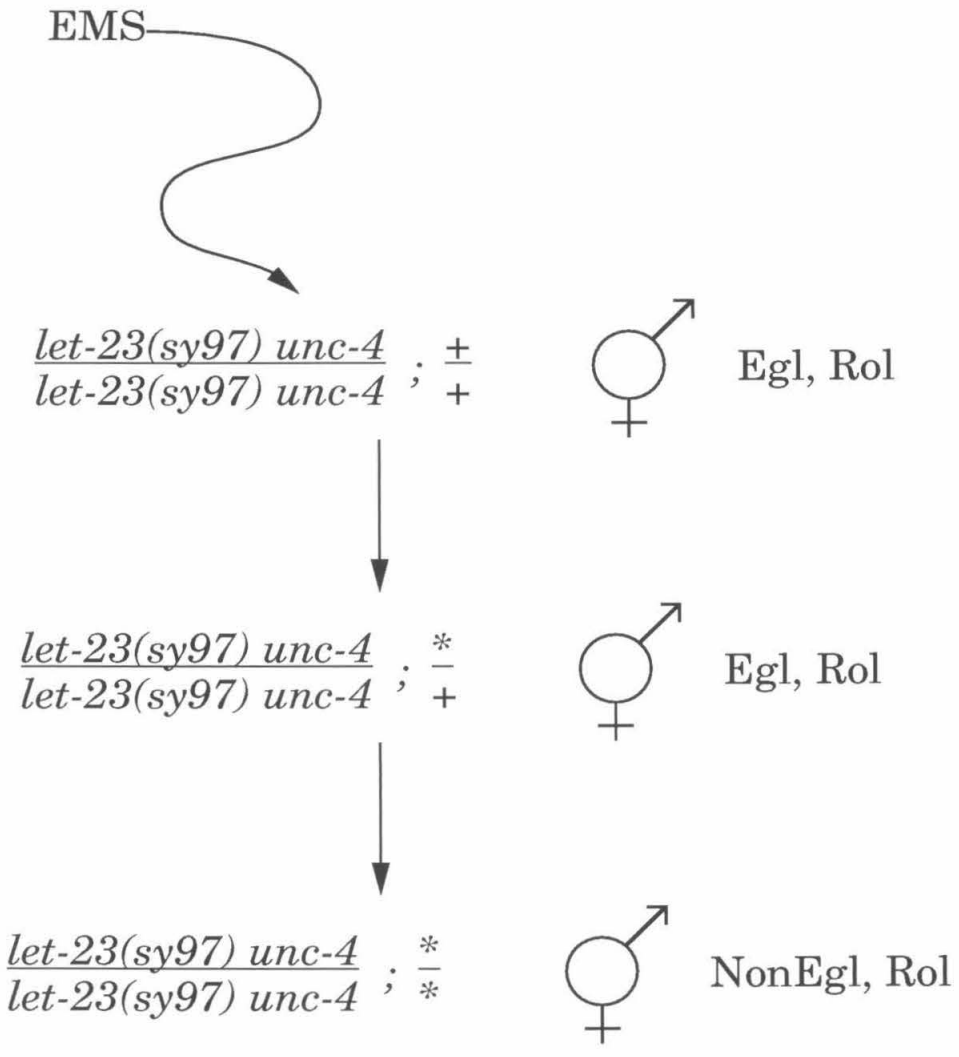
EMS
let-23(sy97) unc-4 / let-23(sy97) unc-4 ; ± / +
Egl, Rol
let-23(sy97) unc-4 / let-23(sy97) unc-4 ; * / +
Egl, Rol
let-23(sy97) unc-4 / let-23(sy97) unc-4 ; * / *
NonEgl, Rol

Figure 2b.

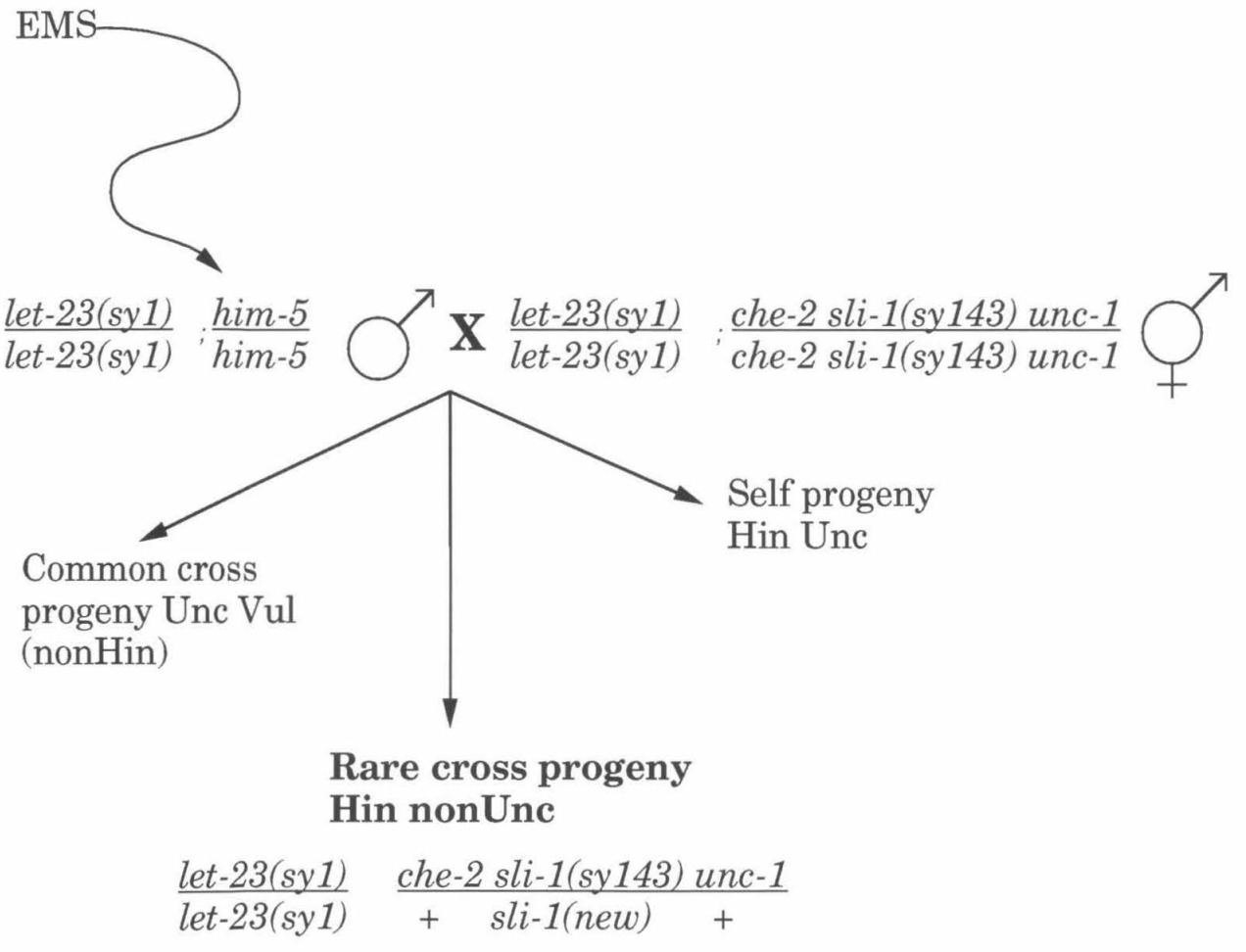
EMS
let-23(sy1) / let-23(sy1) ; him-5 / him-5
X
let-23(sy1) / let-23(sy1) ; che-2 sli-1(sy143) unc-1 / che-2 sli-1(sy143) unc-1
Self progeny
Hin Unc
Common cross
progeny Unc Vul
(nonHin)
Rare cross progeny
Hin nonUnc
let-23(sy1) / let-23(sy1) + che-2 sli-1(sy143) unc-1 / sli-1(new) +

Figure 2c.

Figure 3. Mutations recovered as *let-23* suppressors in the screens described in this paper. Frequency of recovery is only relevant from the reversion of **PS267**, since subviability complicates any estimation of frequency in the other screen.

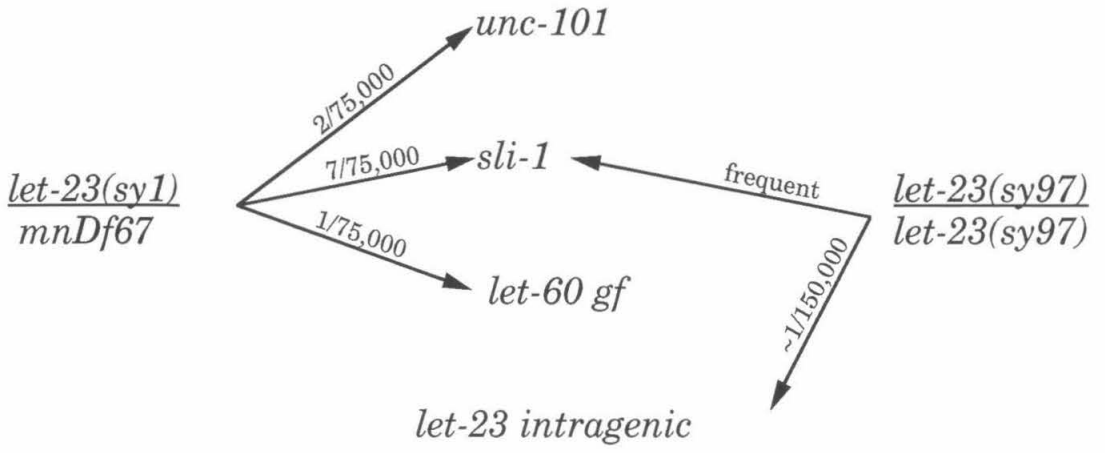

Figure 3. Loci and Frequencies

Figure 4. Genetic map of the left arm of **X** and the markers and rearrangements used in this study.

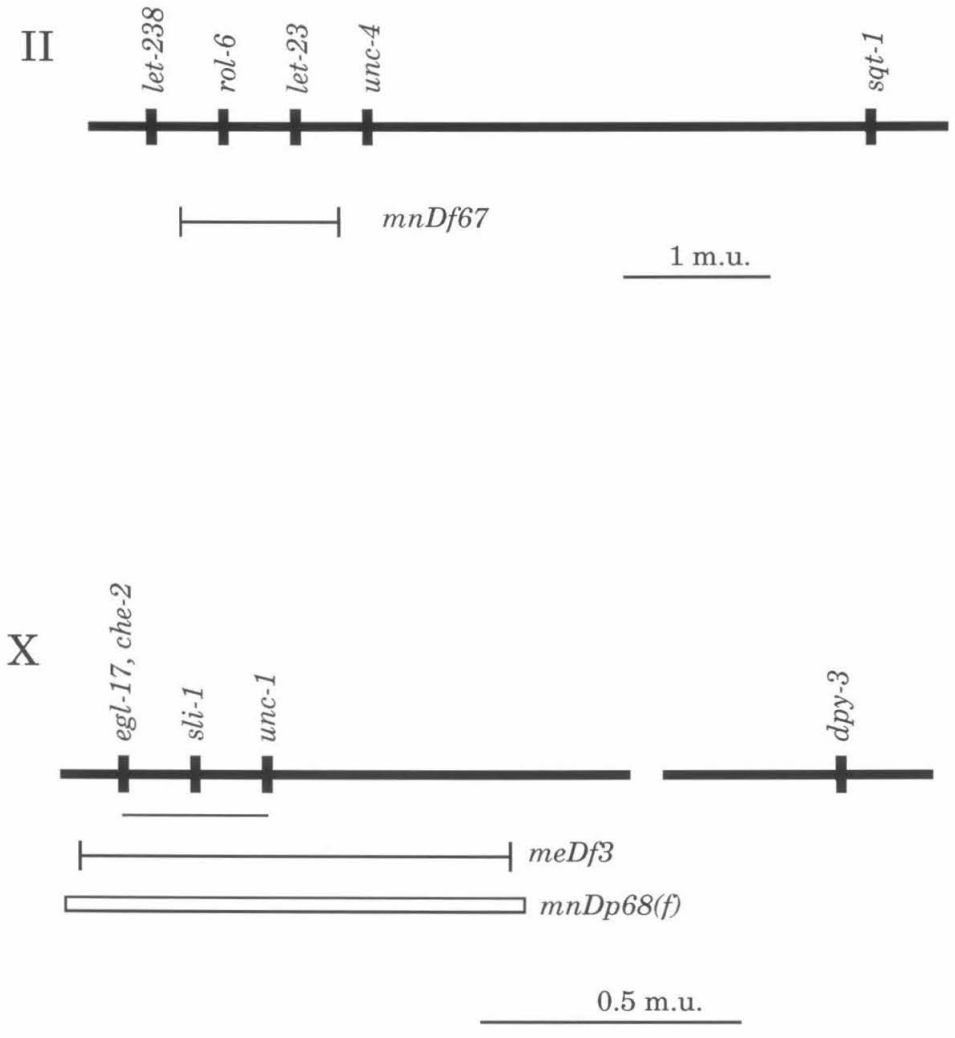

Figure 4. Genetic Maps

Figure 5. Nomarski photomicrographs of L3molt/ young L4 hermaphrodites of various *let-23(sy1)* and *sli-1(sy143)* genotypes. Large black arrowhead marks the anchor cell, white trees show presumed lineage relationships among cells, black trees show presumed lineage relationships among cells that do not normally form vulval tissue. In the *let-23(sy1); sli-1(sy143)* animal, two of the progeny cells of one of the induced VPCs are out of the focal plane. The siblings of these cells are marked by a partial tree.

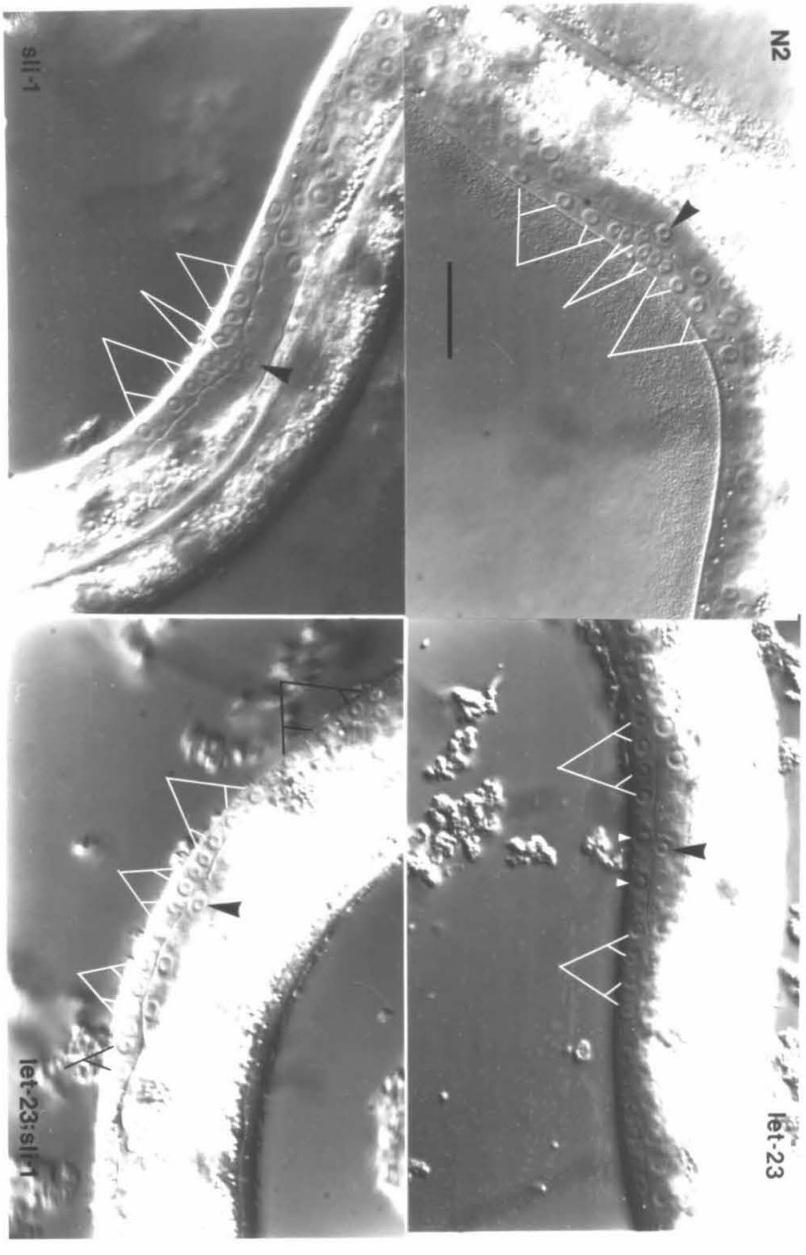
N2
sli-1
let-23
let-23;sli-1

Figure 6. The visible phenotype of *sli-1(sy143).* Nomarski photomicrograph of a L2 *sli-1(sy143)* animal displaying the "notchhead" phenotype present in a small fraction of *sli- I* homozygous larvae.

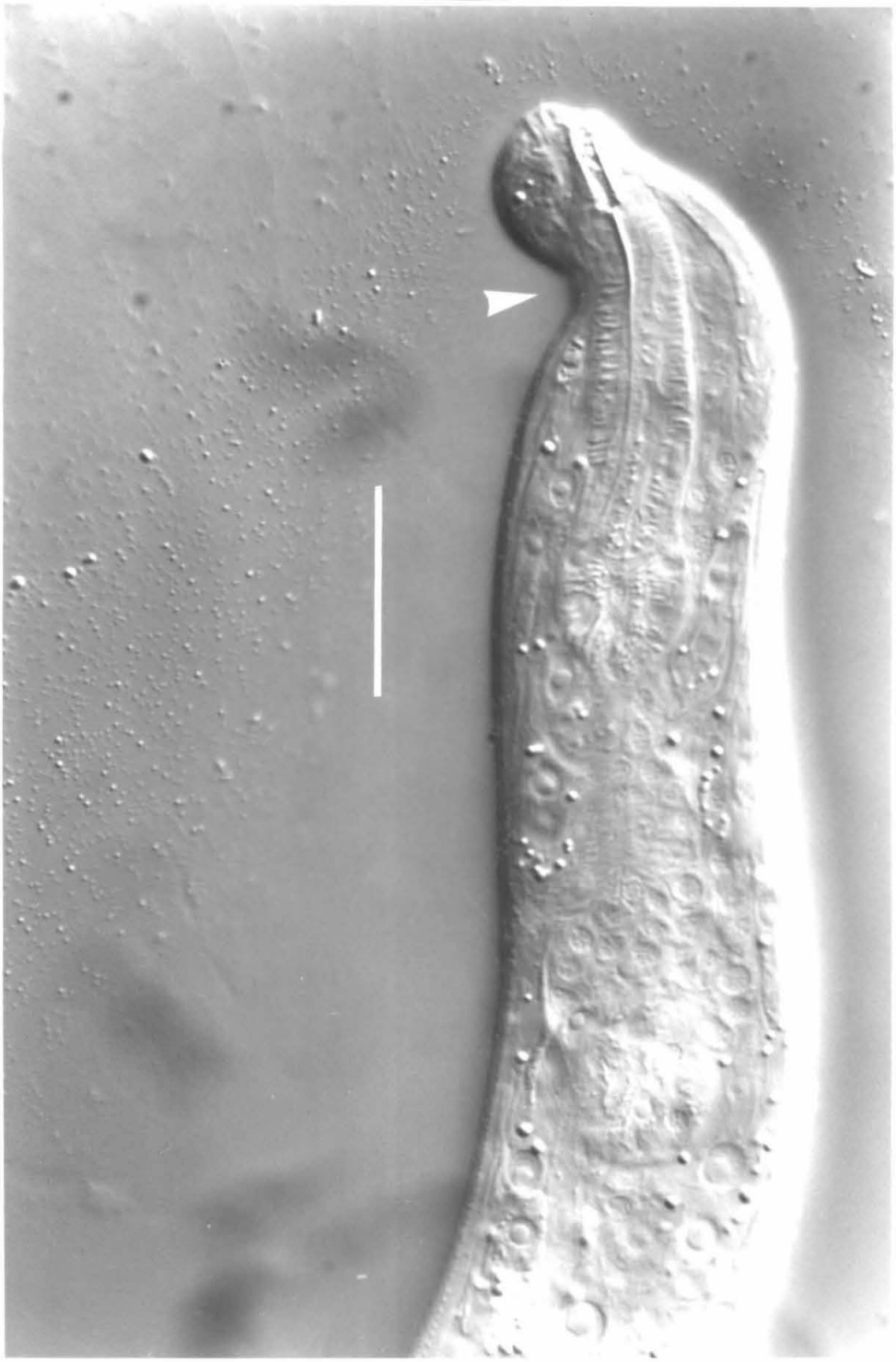

Figure 7. Two potential pathways showing the activity of *sli- 1.*

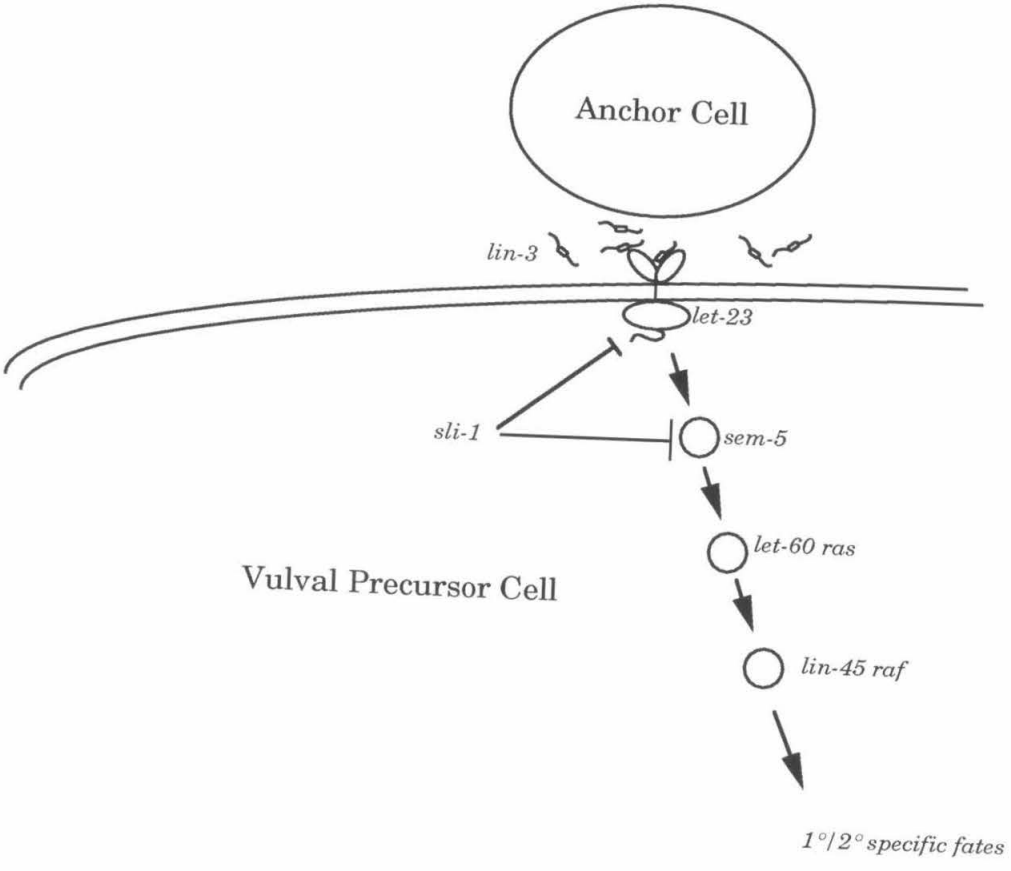
Anchor Cell
lin-3
let-23
sli-1
sem-5
let-60 ras
Vulval Precursor Cell
lin-45 raf
1°/2° specific fates

Figure 7.

Table Legends

Table 1. Extent of vulval differentiation in various *let-23; sli-1* doubly mutant animals. Heterozygous animals were either generated by direct matings or examined under Nomarski optics, removed to petri dishes, allowed to self-fertilize and from progeny phenotypes the maternal genotype was determined.

Table 1. Suppression of the vulval defect of the alleles *let-23(sy1)* and *let-23(sy97)* by various *sli-1* genotypes. % of animals displaying less than 3 VPCs forming vulval tissue, 3 VPCs forming vulval tissue, or greater than 3 VPCs forming vulval tissue, and average # of VPCs forming vulval tissue/animal

	let-23(sy1)				
sli-1 Genotype	%<3	%=3	%>3	n	ave/animal
+/+	95	5	0	29	0.8
+/+/+	100	0	0	22	0.0
sy143/sy143/+	100	0	0	20	0.2
meDf3/+	-	-	-	-	-
sy143/+	91	9	0	23	1.0
sy102/+	100	0	0	10	0.6
sy112/+	100	0	0	13	0.7
sy143/sy143	0	5	95	20	4.3
sy143/meDf3	-	-	-	-	-
sy102/sy102	17	28	55	29	3.7
sy102/meDf3	-	-	-	-	-
sy102/sy143	-	-	-	-	-
sy112/sy112	5	73	23	22	3.2
sy112/meDf3	-	-	-	-	-
sy129/sy129	0	25	75	20	4.3
sy114/sy114	0	55	45	20	3.4
sy115/sy115	35	35	30	20	2.7
sy263/sy263	20	80	0	20	2.5

Table 1. continued.

	let-23(sy97)				
sli-1 Genotype	%<3	%=3	%>3	n	ave/animal
+/+	100	0	0	20	0.0
+/+/+	-	-	-	-	-
sy143/*sy143*/+	-	-	-	-	-
meDf3/+	74	26	0	19	1.3
sy143/+	100	0	0	11	0.05
sy102/+	-	-	-	-	-
sy112/+	-	-	-	-	-
sy143/*sy143*	3	97	0	72	2.9
sy143/*meDf3*	0	100	0	17	3.0
sy102/*sy102*	24	76	0	17	2.5
sy102/*meDf3*	17	83	0	18	2.7
sy102/*sy143*	12	88	0	17	2.9
sy112/*sy112*	-	-	-	-	-
sy112/*meDf3*	68	32	0	22	1.2
sy129/*sy129*	10	90	0	21	2.9
sy114/*sy114*	-	-	-	-	-
sy115/*sy115*	-	-	-	-	-
sy263/*sy263*	-	-	-	-	-

Table 1. Continued

	let-23(+)		
sli-1 Genotype	%=3	n	ave/animal
+/+	100	20	3.0
+/+/+	100	13	3.0
sy143/sy143/+	-	-	-
meDf3/+	100	20	3.0
sy143/+	-	-	-
sy102/+	-	-	-
sy112/+	-	-	-
sy143/sy143	100	20	3.0
sy143/meDf3	100	13	3.0
sy102/sy102	100	20	3.0
sy102/meDf3	-	-	-
sy102/sy143	-	-	-
sy112/sy112	100	20	3.0
sy112/meDf3	-	-	-
sy129/sy129	100	22	3.0
sy114/sy114	-	-	-
sy115/sy115	-	-	-
sy263/sy263	-	-	-

Table 2. Extent of vulval differentiation in doubly mutant animals bearing *sli-1* mutations and mutations in other genes required for vulval induction. In the strain marked with *, the *sli-1(sy102)* allele was used.

1data of Han, Aroian and Sternberg, 1990

Table 2. The ability of *sli-1* mutations to suppress other Vulvaless mutations.

	Average number of VPCs undergoing vulval differentiation per animal			
Vul genotype	*sli-1(+)*	n	*sli-1(sy143)*	n
+	3	20	3	20
lin-3(e1417)	0.8	20	1.4	20
lin-3(n378)	0.8	22	2.0	20
lin-3(n378)/lin-3(n1059)	0.09	20	0.6	10
lin-2(e1309)	0.5	20	4.3	21
lin-2(n768)	2.9	20	3.5	20
lin-7(e1413)	1.0	20	3.3	20
lin-7(n308)	88	20	137	20
lin-10(e1439)	0.5	17	4.1	20
sem-5(n2019)	0.5	20	2.6	23
let-60(sy100 dn)	0[1]	10	0*	16
let-60(n2021 rf)	2.4	20	3.0	27
lin-45(sy96)	0.9	24	1.1	20

Table 3. Extent of vulval differentiation in other animals of genotype *let-23; sli-1*. The data regarding the vulval differentiation of animals of genotype *let-23(sy12); +* is from Aroian and Sternberg 1991.

Table 3. The ability of *sli-1* mutations to suppress other *let-23* alleles.

Genotype	Average number of VPCs undergoing vulval differentiation			
	sli-1(+)	n	*sli-1(sy143)*	n
let-23(n1045) 15°	1.1	20	3.1	20
let-23(n1045) 20°	2.5	20	3.3	20
let-23(n1045) 25°	3.4	20	3.4	20
let-23(sy12) 20°	0.018	31	4.0	20

Table 4. Ability of *sli-1* to suppress other *let-23* mutant phenotypes. Dominant suppression of *let-23(sy97)* lethality by *meDf3* and *sli-1(sy143)* was estimated by comparing the number of animals homozygous for a marker in *trans* to *let-23(sy97)* (either *unc-4* or *rol-6)* to the number of nonUnc or nonRol animals. Suppression of the P12 transformation by either *sli-1(sy143)*/+ or *meDf3*/+ was determined under Nomarski optics followed by confirmation of the genotype of the animal by segregation. Sterility of *let-23(sy12);* + is from the data of Aroian and Sternberg, 1991.

Table 4. Suppression of *let-23* mutant phenotypes in other tissues by *sli-1* mutations.

Genotype		Phenotype(% of animals wild-type)							
let-23	*sli-1*	Essential	n	P12	n	Fertile	n	Spicules	n
sy97	+/+	15	*	73	48	-	-	-	-
sy97	*meDf3*/+	79	191	83	18	-	-	-	
sy97	*sy143*/+	83	952	100	22	-	-	-	-
sy97	*sy143*/*sy143*	102		86	22	-	-	-	-
sy97	+/Ø	ND	-	-	-	-	-	0	
sy97	*sy143*/Ø	ND	-	-	-	-	-	68	19
n1045	+/+	56	75	ND	-	-	-		
n1045	*sy143*/*sy143*	100	27	ND	-	-	-		
sy12	+/+	ND	-	ND	-	0			
sy12	*sy143*/*sy143*	ND	-	ND	-	62	132		
sy15	+/+	0		-	-	-	-		
sy15	*sy143*/*sy143*	0		-	-	-	-		
mn23	+/+	0		-	-	-	-		
mn23	*sy143*/*sy143*	0		-	-	-	-		
mn224	+/+	0		-	-	-	-		
mn224	*sy143*/*sy143*	0		-	-	-	-		

Table 5. Gonad dependence of vulval differentiation in *let-23; sli-1* animals.

Table 5. Gonad dependence of *sli-1* suppression of the *let-23* Vulvaless phenotype

	Average number of VPCs per animal forming vulval tissue			
Genotype	Gonad +	n	Gonad-	n
+;+	3	20	0	many
+; *sli-1(sy143)*	3	20	0	6
let-23(sy1); +	1	20	0	7
let-23(sy1); sli-1(sy143)	4	20	0	5
let-23(sy97); sli-1(sy143)	3	72	0	5

Literature cited

Aroian, R.V., Koga, M., Mendel, J.E., Ohshima, Y. and Sternberg, P.W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature *348*, 693-699.

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Chapter 3

unc-101, a regulator of vulval differentiation acts in many tissues and encodes a clathrin associated protein

Note: Junho Lee and I collaborated on this work. I isolated and characterized the alleles *unc-101(sy108, sy161, sy168, sy169,* and *sy241)*. I constructed and analyzed strains bearing *unc-101* mutations in combination with the genes *let-23, lin-3, let-60, lin-45,* and *lin-15*. I described the lethality associated with *unc-101*mutations. Junho recovered and characterized *sy216*, a potential deficiency of the region. He also constructed and analyzed animals bearing mutations at *unc-101* and *lin-2, lin-7,* and *lin-10*. All molecular work was done by Junho.

unc-101, a gene required for many aspects of C. elegans development and behavior, encodes a clathrin-associated protein.

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SUMMARY

Our genetic analysis indicates that the C. elegans *unc-101* gene is required for many aspects of development and behavior, including negative regulation of vulval induction. We have cloned *unc-101* and found that it encodes a homolog of the mammalian AP47 and AP50 genes, medium chains of clathrin-associated protein complexes located at the trans-Golgi and the plasma membrane, respectively. Comparison of these sequences to the full length sequence of a C. elegans AP50 homolog reveals that Unc-101 is most closely related to mammalian AP47. Mammalian AP47 and nematode Unc-101 are functionally equivalent as assayed in transgenic nematodes. C. elegans thus provides a genetic system in which to study clathrin-associated proteins.

Introduction

Clathrin coated pits and coated vesicles are organelles that originate from the plasma membrane and the trans-Golgi in eukaryotic cells and mediate intracellular trafficking of membrane proteins (Fig 1a; reviewed in Brodski, 1988; Keen, 1990; Pearse and Robinsons, 1990). The coated vesicles are composed of two units, clathrin triskelion cages and their associated protein (AP) complexes. While clathrin triskelions are common structural units to both the plasma membrane and the trans-Golgi, the AP complexes differ in these compartments, probably conferring specific functions to differentially located clathrin vesicles. Cell fractionation studies (Ahle et al., 1988; Keen, 1987) showed that the trans-Golgi associated protein complex AP-1 consists of two large chains, β' and γ, one medium chain, AP47, and one small chain, AP19 (Fig. 1b). The plasma membrane associated protein complex AP-2 consists of two large chains, α and β, one medium chain,

AP50, and one small chain, AP17. While the large chains β and β' share common amino acid residues, the large chains α and γ have unique primary structure. Both medium chains and small chains are homologous to their counterparts.

The coated vesicles of the plasma membrane are involved in the endocytosis of membrane proteins such as LDL receptor, transferrin receptor, T cell receptor, and EGF receptor (references found in Keen, 1990; Pearse and Robinsons, 1990). Trans-Golgi coated vesicles are involved in sorting of proteins such as lysosomal enzymes. Trans-Golgi coated vesicles are also thought to be involved in regulated secretion or transport of proteins to the plasma membrane.

Genetic analysis of clathrin coated vesicles in Saccharomyces cerevisiae showed that yeast mutants deficient in the clathrin heavy chain are defective in the retention of the endonuclease Kex2p protein in the Golgi apparatus (Seeger and Payne 1992a, Seeger and Payne 1992b). Although biochemical studies in mammalian cells suggest that the AP complexes of the clathrin coated vesicles may have essential functions in cells, it does not seem to be true in the yeast cells. Deletion of yeast homologs of the small chains of the APs has no effect on cell growth, protein export, or endocytosis (J, Finlay, personal comm.). Similarly, the deletion of a yeast homolog of medium chains does not result in any obvious phenotypic consequences (S.K. Lemmon, personal comm.). Since yeast is a single-cell organism and yeast clathrin vesicles may have fewer functions than those in multicellular organisms, it is useful to examine the functions of the proteins in the multicellular organisms to understand the function of the coated vesicles and their APs.

The *unc-101* locus of C. elegans was originally identified by virtue of its uncoordinated movement (D. Riddle, personal comm.), and independently by abnormal uptake of dye by sensory neurons (E. Hedgecock, personal comm.). We

identified unc-101 alleles as extragenic suppressors of a reduction-of-function mutation of *let-23*, an epidermal growth factor receptor-like tyrosine kinase (G. Jongeward and P. Sternberg, in prep). Here, we determine the loss of function phenotype of *unc-101*, and its role in vulval induction. We describe the cloning of *unc-101*, its sequence, and its functional equivalence with the murine AP47 protein. C. elegans thus provides an opportunity to study molecular genetics of functions of clathrin-associated protein complexes in a multicellular organism.

Results

Pleiotropic effects of *unc-101* mutations

unc-101 mutations have pleiotropic effects on the behavior and development of the nematode C. elegans. *unc-101* animals have uncoordinated movement. They are very sluggish, do not respond to a light touch, and tend to coil. *unc-101* animals also display abnormal uptake of the dye FITC (E. Hedgecock, personal comm). In wild-type animals six pairs of neurons in the amphid and two pairs of neurons in the phasmid are filled with FITC (Hedgecock, et al., 1985). In *unc-101* animals, only one pair of amphid neurons stains brightly, and the phasmid neurons are generally faint or unstained. *unc-101* mutant animals show irregularity in their defecation cycles (Thomas, 1990). We have also isolated *unc-101* mutations as suppressors of the *let-23(sy1)* vulvaless phenotype (this study; Jongeward and Sternberg, in prep).

unc-101 animals also have male tail defects such as abnormal ray and spicule structure (S. Emmons, H. Chamberlin, personal comms.). In addition, about half of *unc-101* animals do not survive to reach adulthood. These inviable animals arrest in late L1 stage, and in few cases in L2, L3, or L4 stage.

Isolation of null alleles of unc-101

The *unc-101* alleles *m1, rh6, sy108*, and *sy161* had previously been recovered from independent F2 screens for the different phenotypes of *unc-101* mutations. *m1* was isolated in a screen for animals displaying uncoordinated (Unc) movement (D. Riddle, personal comm.), *rh6* in a screen for animals displaying uncoordinated behavior and amphid FITC staining defect (E. Hedgecock, personal comm.), and *sy108* and *sy161* in a screen for suppressors of the egg-laying defect of *let-23(sy1) / Df* animals (G. Jongeward and P. Sternberg, in prep). Although they were recovered in screens for different phenotypes, animals homozygous for these alleles display essentially identical phenotypes to those described above.

Since the F2 screens could have failed to recover the null alleles of *unc-101* had the null phenotype been lethal, we performed several genetic screens that could recover null alleles of *unc-101*(Fig. 2). We first screened for new alleles that failed to complement the Unc phenotype of *unc-101(sy108)* (non-complementation screen). Null alleles can be isolated in this screen because some animals carrying unc-101(sy108)/ in trans to a deletion of the unc-101 locus are viable. Two alleles, *sy168* and *sy169*, were recovered in a screen of 15,000 EMS-mutagenized F1 gametes. The phenotypes animals homozygous for these two alleles were essentially identical to those of other alleles.

We recovered *sy216* by virtue of its failure to complement *unc-101(rh6)* in a similar non-complementation screen of 11,000 trimethylpsoralen (TMP)-mutagenized gametes. Animals homozygous for *sy216* arrest immediately after hatching without any apparent post embryonic divisions. These animals live for several days as arrested L1 stage larvae before finally dying. This arrest phenotype is different from that associated with other *unc-101* alleles, since dying homozygotes of other alleles arrest and die rapidly as late L1 larvae (and rarely as L2, L3, or L4

stage larvae). The lethality of *unc-101(sy216) / unc-101(sy108)* trans-heterozygotes is slightly enhanced: 74% of *sy216/ sy108* heterozygous animals were inviable while 45% of *sy108/ sy108* animals were inviable. However, suppression of the *let-23(sy1)* mutation was not enhanced, since *sy216/ sy108*; *let-23(sy1)* animals averaged 3.6 VPCs undergoing vulval differentiation, which is the same level of vulval differentiation of *sy108 / sy108 ; let-23(sy1)* animals (n= 20, respectively). Since TMP is known to induce high frequency of small deletions (L. Edgar, personal comm.), rearrangements that delete neighboring genes as well as *unc-101* could have been recovered. To address this issue genetically, we constructed the trans-heterozygote *unc-101(sy216) / eDf3. eDf3* fails to complement the mutations that define the nearest genetically defined loci to the right, *unc-59 , let-201, let-202,* and *let-203.* This heterozygote is viable and wild-type. Therefore *sy216* does not delete the nearest genetically defined loci to the right. *sy216* does not delete the nearest gene to the left, *ced-1*: An animal of genotype *dpy-5 + sy216 / hInI* was found in the cross-progeny of a + *ced-1 sy216 / dpy-5 + unc-101(rh6)* hermaphrodite mated with *hInI* males. If the *ced-1* region had been deleted, no recombination could have occurred in this region. However, it is likely that there are essential gene(s) in the interval between *ced-1* and *eDf3,* as this region is not saturated for lethal mutations. It is probable that *sy216* deletes not only the entire *unc-101* locus, but also essential gene(s) nearby.

The non-complementation screens described above could have missed some of new mutations because about half of the progeny from homozygous *unc-101* mothers die. We therefore designed another screen to avoid loss of new alleles due to subviability by providing a wild-type maternal copy of *unc-101*(Fig. 2c). All animals of the genotype *unc-101(sy108) / unc-101(sy216)* from a mother of the genotype *unc-101(sy108) / +* are viable. Therefore, the maternal copy of *unc-101*(+) is sufficient

to rescue the inviability of any new allele in trans to the visible allele *sy108*. Two EMS induced alleles, *sy241* and *sy242* were recovered from a screen of 20,000 F1 mutagenized gametes. *sy241* animals are viable and have phenotypes similar to those of animals of previously identified alleles. *sy242* was lost before it could be analyzed.

We conclude that the phenotypes represented by homozygotes of viable alleles are those associated with null alleles. We base this conclusion on two genetic arguments. First, the frequency of recovery is consistent with that of loss-of-function alleles (e.g., Brenner, 1974; Greenwald and Horvitz, 1980). Second, homozygotes of these alleles are phenotypically indistinguishable. The exception to the second criterion is *sy216*, which we believe is a deletion of *unc-101* locus itself and adjacent gene(s). As discussed below, our molecular analysis is consistent with this argument.

***unc-101* is a negative regulator of the vulval induction pathway.**

Two alleles of *unc-101* (*sy108* and *sy161*) were recovered as suppressors of the Vulvaless (Vul) phenotype of the weak *let-23* allele, *sy1*. *let-23* is a C. elegans homolog of the epidermal growth factor (EGF) receptor tyrosine kinase that is involved in the development of several structures including the hermaphrodite vulva. Several non-null mutations of *let-23* are suppressed (Table 1). *let-23(sy1)*, the allele used in the original screen is suppressed strongly. *let-23(sy1)* animals average one VPC per animal forming vulval tissue, while *unc-101; let-23(sy1)* animals are hyperresponsive to the signal, averaging 3.6 VPCs forming vulval tissue per animal. Mutations at the *unc-101* locus confer no defect in the extent of vulval differentiation in the absence of another mutation, averaging three VPCs forming vulval tissue per animal. A more severe, but not a null, mutation, *let-*

23(sy12) is suppressed from an average of less than one to nearly three VPCs per animal forming vulval tissue. Another severe allele, *let-23(sy97)*, is suppressed but only to a very slight extent. In contrast to suppression of these alleles, the allele *let-23(n1045)* is enhanced by an *unc-101* mutation. For instance, at 20°, *let-23(n1045)* animals average 2.5 VPCs forming vulval tissue, while *unc-101; let-23(n1045)* animals average only 0.7 VPCs forming vulval tissue. This enhancement holds true at all temperatures tested.

let-23 acts in several tissues (Aroian and Sternberg, 1991). *unc-101* mutations suppress *let-23* mutant phenotypes in a subset of these tissues. Approximately 85% of *let-23(sy97)* animals die as L1 larvae. This lethality is not suppressed by an *unc-101* mutation (data not shown). True null alleles of *let-23* confer L1 larval lethality on all homozygotes. An *unc-101* mutation will not suppress this lethality, although it is possible that a few *unc-101; let-23(null)* animals are viable (data not shown). The sterility of the allele *let-23(sy12)* is partially suppressed. While all viable *let-23(sy12)* hermaphrodites are sterile, approximately 65% of *unc-101; let-23(sy12)* animals are at least slightly fertile.

To determine if the interaction of *unc-101* was limited to *let-23* or if *unc-101* mutations were capable of suppressing mutations of other genes required for vulval induction, we constructed a series of double mutants and examined the extent of vulval differentiation in these animals (Table 1). *lin-2, lin-7,* and *lin-10* are genes that act near *let-23* and are required for vulval induction (fig. 3). *lin-2, lin-7,* and *lin-10* mutants have defects exclusively in the vulval induction process (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson et al., 1987; Sternberg and Horvitz, 1989; Kim and Horvitz, 1990). Strong reduction-of-function alleles of any of these loci cause a vulvaless phenotype with an average of 0.4 to 0.9 VPCs per animal undergoing vulval differentiation. *unc-101* mutations

can suppress these vulvaless phenotypes to an average of 3.4 VPCs differentiating per animal.

lin-3 encodes a member of the EGF family of signaling molecules and is most likely the inductive signal (Hill and Sternberg, 1992). Reduction-of-function mutations at this locus are suppressed only partially by an *unc-101* mutation. Specifically, *lin-3(n378)* homozygotes average less than one vulval precursor cell (VPC) per animal forming vulval tissue, while *unc-101; lin-3(n378)* double mutants average slightly more than two VPCs forming vulval tissue per animal (three VPCs form vulval tissue in wild-type animals). This partial suppression is also true for animals bearing the allele *lin-3(e1417)*, although the extent of suppression is not as great.

let-60 is a *ras* homolog in C. elegans. Loss-of-function mutations or dominant negative mutations of this locus result in lethality and a vulvaless phenotype (Han, et al., 1990, Han and Sternberg, 1990). Dominant negative mutations of the *let-60 ras* gene are not suppressed by *unc-101.* Neither animals of genotype *let-60(dn)* nor *unc-101; let-60(dn)* display any vulval differentiation.

lin-45 is a raf homolog that likely acts downstream of *let-60* (Han et al, 1993). A reduction-of-function mutation of *lin-45 raf* is partially suppressed by an *unc-101* mutation. *lin-45(sy96)* animals average one VPC forming vulval tissue, while *unc-101; lin-45(sy96)* animals average 1.9 VPCs forming vulval tissue.

To summarize, *unc-101* mutations suppress reduction-of-function mutations of many of the genes required for vulval differentiation. Mutations at the *lin-2, lin-7,* and *lin-10* loci are suppressed to an extent similar to that seen with weak *let-23* alleles. From these data, we conclude that *unc-101* is a negative regulator of vulval induction, required for the proper regulation of EGF-Receptor mediated signaling (Fig. 3). However, *lin-3* (the putative inductive signal, Hill and Sternberg, 1992)

and *lin-45 raf* (Han, et al. 1993) are only partially suppressed. *let-60 ras* dominant negative mutations are not suppressed. Therefore we propose that *unc-101* acts at or near the *let-23* step. The fact that *unc-101* mutations do not suppress any complete loss of function mutations suggest that *unc-101* mutations cannot bypass the lack of the molecules in the signalling pathway such as the Let-23 receptor tyrosine kinase or the Let-60 *ras* protein.

Correlation of the genetic and physical maps near *unc-101*

To clone *unc-101*, we correlated the genetic and physical map around *unc-101* by identifying and mapping transposon polymorphisms close to *unc-101* (Fig. 3). Multipoint restriction fragment length polymorphism (RFLP) mapping (Ruvkun et al., 1989) was used to map two transposon polymorphisms, TCUNC101A and TCUNC101E, with respect to the genetic markers *unc-75*, *ced-1*, *unc-101* and *unc-59*. The source of these RFLPs was the strain MT3618 (*unc-75 ced-1 unc-59*), which contains a transposon rich region between *unc-75* and *unc-59* (S. Glass, T. Gerber and R. Horvitz, personal comm.), relative to the *unc-101 (sy108)* strain, a typical Bristol strain. We recovered Unc-59 non Unc-75 recombinants from + + *unc-101(sy108)* + / *unc-75 ced-1* + *unc-59* heterozygotes. By inverse PCR of genomic DNA from different recombinant animals (Ochman et al., 1988), we identified the flanking region of two polymorphisms, TCUNC101A and TCUNC101E. We found that TCUNC101A was present in the congenic strain, but was not present either in the + + + *unc-59* or the + + *unc-101 unc-59* recombinants, indicating that this polymorphism is to the left of *unc-101* on the genetic map. TCUNC101E was present in + + + *unc-59*, but not in + + + unc-*101 unc-59* recombinants; therefore, TCUNC101E is near *unc-101* and to the right of TCUNC101A.

We further mapped these two polymorphisms physically and genetically. By hybridization to a yeast artificial chromosome (YAC) grid filter (Coulson et al., 1991), we located TCUNC101A on the physical map on the right arm of chromosome I. TCUNC101E was located about 600 kb to the right of TCUNC101A on the same contig. To genetically map these polymorphisms, we recovered 75 more recombinants from the heterozygotes described above, and performed either Southern hybridization with TCUNC101E as probe or PCR with TCUNC101E primers. No recombination events were observed between TCUNC101E and *unc-101*, indicating that TCUNC101E is very close to *unc-101*. Using these recombinants, the relative genetic distance between *ced-1*, *unc-101*, and *unc-75* could be more precisely defined. The relative frequency of recombination was *unc-75* (5/38) *ced-1* (12/38) *unc-101* (21/38) *unc-59*. The other 37 recombinants were recovered in a screen biased to detect recombination between *unc-75* and *unc-101*. Since the physical distance between the two polymorphisms is about 600 kb, and since TCUNC101E is inseparable from *unc-101*(+) marker, we proceeded to test genomic cosmids within 100 kb of TCUNC101E for the ability to complement the *unc-101* phenotype.

Rescue of *unc-101* mutations by DNA-mediated transformation

We identified a genomic cosmid that can rescue the uncoordinated phenotype of *unc-101* mutations by DNA-mediated transformation. We tested five cosmids within 100 kb of TCUNC101E for their ability to rescue the uncoordinated phenotype of *unc-101(sy108)* by introducing cosmid DNA as an extrachromosomal multicopy transgene (Fig. 4; Mello et al., 1990). Only cosmid W05A3 was able to rescue *unc-101(sy108)*. A 6.3 kb subclone of W05A3, pJL5, is the smallest genomic fragment capable of rescuing the uncoordinated phenotype. The pJL5 subclone

rescues at least three other phenotypes of *unc-101(sy108)*: lethality, defective uptake of FITC, and suppression of *let-23(sy1)* vulvaless phenotype. The partial lethality of *unc-101(sy108)* was rescued in the transgenic animals carrying pJL5 (Table 2). The viability of non-transgenic animals is 47%. 72% of the progeny of the transgenic parents were viable. pJL5 also suppresses the FITC staining defect of *unc-101(sy108)* animals (data not shown). The vulval differentiation of the transgenic animals of *unc-101 (sy108)*; *let-23 (sy1)* ; *Ex[pJL5]* was lowered to 0.8 VPCs induced per animal (n=20), which is the level of *let-23 (sy1)* single mutant animals but unlike the vulval differentiation of *unc-101(sy108)*; *let-23(sy1)* double mutant animals (3.6 VPCs per animal). Therefore, pJL5 also rescues the suppression of the *let-23(sy1)* mutation. We did not test the rescue of the other phenotypes associated with *unc-101* mutations.

The inviability of animals homozygous for *sy216* was not rescued by the W05A3 cosmid, indicating that *sy216* might delete other essential gene(s) near *unc-101* (see Experimental Procedures).

Genomic and cDNA structure of *unc-101*

We isolated a full length cDNA clone from a cDNA library (Barstead and Waterston, 1989) using the pJL2 plasmid as probe (Fig. 4), and determined its nucleotide sequence (Fig 5a). There are three in-frame stop codons 5' to the putative ATG codon. Also, the 5' end of the cDNA sequence has nine nucleotides that are identical to the 3' end sequence of the trans-spliced leader sequence SL1 (Krause and Hirsh, 1987) indicating that this cDNA has a full length coding sequence and that this gene is a trans-spliced gene. A polyadenylation signal, AATAAA, is present at nucleotide 1844. The nucleotide sequence predicts that the *unc-101* gene product is a protein of 422 amino acids.

We inferred the genomic structure of *unc-101* by partial genomic sequence data and PCR with primers specific to cDNA regions (Fig 5b). *unc-101* consists of seven exons separated by six introns. The smallest rescuing plasmid, pJL5, has 95 nucleotides 5' to the SL-1 acceptor sequence.

Physical basis of *unc-101* mutations

To confirm our identification of the *unc-101* coding region, we analyzed the lesions associated with *unc-101* alleles. We determined the locations of the mutations of eight alleles of *unc-101* (Fig 5b). We determined the mutations by directly sequencing PCR-amplified DNA preparations from genomic DNA or single mutant animals (Kretz et al., 1989). All but one mutation are predicted to result in truncated proteins due to either deletions or nonsense mutations. *sy108* is a deletion of 115 nucleotides in exon 3 and intron 3 and an insertion of 8 nucleotides at the deletion point. *sy168* and *sy169* , which were obtained in the non-complementation screen using *sy108,* were the same mutation as *sy108*, suggesting that these are results of gene conversion events, or recovery of the maternal allele. *sy237, sy241*, *m1* and *rh6* are nonsense mutations, encoding truncated proteins. *sy237* is a G to A transition at the nucleotide 550, making TGG to TAG stop codon. *sy241* is a C to T transition mutation at the nucleotide 1284, changing CAA to TAA stop codon. *m1* is a C to T mutation at the nucleotide 1314, changing CAA to TAA stop codon. *rh6* is another C to T mutation at the nucleotide 1086, changing CAA to TAA. *sy161*, the only missense mutation, is a C to T mutation at the nucleotide 552, changing CGC (arginine) to TGC (cysteine). This arginine residue is conserved in both AP47 and AP50 (see results below). We were unable to amplify by PCR any genomic DNA from *sy216* homozygotes, suggesting that this mutation is a deletion of the entire gene (see Experimental Procedures).

***unc-101* encodes a clathrin-associated protein.**

A database search with the translation of the *unc-101* cDNA sequence identified very high similarity to two mammalian proteins, AP47, the medium chain of the trans-Golgi associated clathrin-associated complex AP-1, and AP50, the medium chain of plasma membrane associated clathrin-associated complex AP-2(Fig. 7; Nakayama et al., 1991; Thurieau et al., 1988) Unc-101 is also similar to a yeast protein, Yap54, the yeast homolog of AP47 (Nakayama et al., 1991). Unc-101 protein is 74% identical to mammalian AP47, and 42% to mammalian AP50, suggesting that *unc-101* is a homolog of the mammalian AP47.

To confirm that Unc-101 is a homolog of AP47 protein, we sought to identify AP50 homologs in C. elegans. The C. elegans genome sequencing consortium has identified a cDNA encoding a homolog of AP50 (Waterston et al., 1992). Using this cDNA clone as probe, we isolated three more cDNA clones and determined their sequences. All three encoded a single gene. One of the three cDNA clones had a full length coding sequence (Fig 6). The comparison of the amino acid sequence of this protein with those of other homologs revealed that this AP50 homolog protein is 81% identical to mammalian AP50 and 40-42% identical to the AP47 homologs, indicating that this sequence is indeed an AP50 homolog (Fig. 7). Based on the extent of the amino acid sequence identity, we believe that *unc-101* encodes a C. elegans homolog of AP47 protein, medium chain of the trans-Golgi associated clathrin-associated protein complex.

We physically mapped the AP50 homolog to chromosome X by YAC grid hybridization, and identified a cosmid (T16D11) that contains the entire genomic region of AP50 homolog in the middle of its insert (data not shown). When injected into *unc-101 (sy108)* animals, this cosmid did not rescue the Unc phenotype of *unc-*

101 (sy108) animals. This negative result is consistent with *unc-101* being a homolog of AP47, but not AP50.

The function of AP47 clathrin-associated protein is conserved in mammals and nematodes.

Since the amino acid sequence of *unc-101* is similar to that of the mammalian homolog AP47, it is conceivable that their function is conserved during evolution. To test this hypothesis, we examined whether the mammalian AP47 homolog can rescue the mutant phenotype of *unc-101* animals. We constructed a hybrid gene with *unc-101* genomic DNA and murine AP47 cDNA (Fig. 8a). In the mouse/nematode hybrid construct, 298 amino acid residues of 422 amino acids are from the murine cDNA. We also constructed an *unc-101* cDNA hybrid gene that is identical to the AP47 hybrid except that it has *unc-101* cDNA portion instead of AP47 cDNA. Both of these hybrid genes rescue the Unc phenotype of *unc-101(sy108)* animals (for instance see fig. 8b). The level of vulval differentiation of the transgenic *unc-101(sy108)*; *let-23(sy1)* animals carrying the AP47 hybrid gene was average of 2.0 VPCs per animal, compared with 3.6 VPCs of *unc-101(sy108)*; *let-23(sy1)* animals without the transgene, indicating that the suppression of the *let-23(sy1)* vulvaless phenotype was also rescued (Fig. 8c). Thus, mammalian AP47 and C. elegans *unc-101* are not only very similar in sequence, but also functionally equivalent. Rescue of the subviability of *unc-101(sy108)* animals was not checked due to low transmission of the transgene.

Discussion

We have analyzed the C. elegans *unc-101* gene genetically and molecularly. We demonstrated that *unc-101* mutations suppress the vulvaless phenotype of weak

alleles of let-23, a *C. elegans* EGF receptor homolog required for vulval induction. We have cloned the *unc-101* gene and shown that it encodes a homolog of AP47, the medium chain of the trans-Golgi associated clathrin-associated complex. We also determined a full length sequence of a homolog of AP50, the medium chain of plasma membrane associated complex. The DNA and amino acid sequence comparison clearly showed that *unc-101* is an AP47 homolog. This homology was confirmed by the fact that a nematode *unc-101*/ mammalian AP47 hybrid gene could functionally replace the nematode *unc-101* gene.

Null phenotype of *unc-101*

We analyzed alleles of *unc-101* recovered in several screens to define the null phenotype of this locus. We are convinced that the visible alleles represented by *sy108* are strong reduction-of function if not null alleles of *unc-101* for the following reasons. First, the non-complementation screens we performed should be able to recover null alleles of *unc-101* and the recovery frequency was consistent with that for typical null alleles (Brenner, 1974; (Greenwald and Horvitz, 1980)). Second, with the exception of *sy216,* all alleles recovered from non-complementation screens display very similar phenotypes to the previously recovered alleles. The lethal phenotype of *sy216* is quite different from that of other alleles, indicating that this lethality is due to a mutation in other essential gene(s) near *unc-101.* Supporting this possibility are the facts that we were unable to amplify any *unc-101* genomic DNA from *sy216* homozygotes, that the rescuing cosmid could not rescue *sy216* despite its ability to rescue the lethality of *unc-101(sy108)*, and that the Southern analysis failed to show any polymorphism in *sy216* / + heterozygotes using the rescuing cosmid as probe (data not shown), indicating that the deletion of *sy216* is bigger than 30 kb. Third, sequence analysis of the mutant alleles showed that all

but one allele are deletions or nonsense mutations, encoding truncated, and probably non-functional proteins.

The subviability of the putative null alleles of *unc-101* could be explained by the presence of a partially redundant homolog of AP47. This redundant homolog could be expressed at different levels in different individual animals, and a higher level of expression might take over some essential function of *unc-101* protein, enabling them to survive despite loss of *unc-101*. It is also formally possible that the N-terminal residues of the predicted truncated mutant proteins provide some essential function.

Structure and function of AP50/ AP47 proteins.

It is not surprising that AP50 and AP47 sequences are similar (40% identical over 422 amino acids). Other components of the clathrin-associated complexes also display similarity. Mammalian AP17 and AP19 small chains have 44% identity, and β and β' heavy chains show the highest degree of similarity of 84% (Kirchhausen et al., 1991) . The exception is α and γ heavy chains, which do not share much homology. Given the extent of homology between the components of the APs, it is conceivable that β and β' chains will have common functions such as binding to clathrin units, and that the medium chains and small chains have some specific and some common functions. In contrast, α and γ heavy chains may have specific functions such as binding to specific membrane marker protein or membrane receptor proteins.

Amino acid residues conserved in both AP47 and AP50 may have common molecular functions such as interaction with clathrin units and membrane components. These residues are scattered throughout the peptide sequence. Amino acid residues specifically conserved in either AP47 or AP50 homologs may be

important for their specific functions such as interaction with specific membrane proteins, and/or other components of their own type of associated complex. These residues are also scattered throughout the peptide sequence, making it difficult to predict the domain structure of the proteins.

We had hoped to find important residues for AP47-specific function by determining the locations of *unc-101* mutations. However, most alleles are deletions or nonsense mutations. *sy161* is the only missense mutation, and it occurs at an amino acid conserved in both the AP47 and AP50 proteins. Therefore, we were unable to define any amino acid residues important for AP47-specific function. To identify such residues, more extensive screens for *unc-101* mutations could be performed using the protocols we described here.

The *m1* and *rh6* alleles encode proteins missing just a few C-terminal residues but confer identical phenotypes to other alleles. It is not clear why these alleles behave as severe as other alleles, but it is possible that the C-terminal residues are important for its function, stability, or essential regulation. Only one of eight mutations being a missense mutation is surprising, as high identity among homologs is generally assumed to imply that these conserved residues are functionally important.

Negative regulation of C. elegans vulval induction

In C. elegans, a signal from the anchor cell (AC) of the somatic gonad induces three out of six vulval precursor cell (VPC)s to generate vulval tissue. Proper vulval development requires gene functions for both the induction of vulval tissue cells and for the prevention of excessive induction. Many genes required for the production of vulval tissue have been genetically and molecularly characterized and shown to encode homologs of mammalian signaling molecules (reviewed by Horvitz and

Sternberg, 1991). The inductive signal produced by the anchor cell is likely to be encoded by *lin-3* (Hill and Sternberg, 1992). The signal encoded by *lin-3* is likely to be received by the *let-23* gene product, an EGF receptor homolog (Aroian et al., 1990). The genes *sem-5* and *let-60*, proposed to act downstream of *let-23*, encode a GRB2 homolog with SH2 and SH3 domains, and a *ras* homolog, respectively (Clark et al., 1992; Han and Sternberg, 1990). *lin-45*, which likely acts after *let-60*, encodes a C. elegans *raf* homolog (Han et al., 1993). Reduction-of-function mutations in these genes result in a vulvaless (Vul) phenotype. The other genes, *lin-2, lin-7,* and *lin-10* , are also required for vulval induction.

There are a number of genes acting as negative regulators of the induction pathway. Loss-of-function mutations of *lin-15* cause a gonad-independent multivulva phenotype (Ferguson and Horvitz, 1985; Ferguson et al., 1987; L. Huang, P. Tzou, P. Sternberg, unpublished results). *lin-15(+)* activity from cells other than anchor cell or VPCs antagonizes the inductive signal (Herman and Hedgecock, 1990). Here we have shown that *unc-101* also acts as a negative regulator of this process. Mutations at *unc-101* suppress defects associated with mutations in genes such as *let-23, lin-2, lin-7* and *lin-10* that are required for specification of vulval fates, suggesting that the wild-type function of *unc-101* is to negatively regulate the process of vulval induction. However, the loss of *unc-101* activity in an otherwise wild-type animal confers no vulval differentiation defect (*unc-101* homozygotes, however, do have defective vulval morphogenesis, resulting in an egg-laying defect), suggesting that *unc-101* acts to refine the response to the inductive signal, rather than to prevent cells from generating vulval cells. Consistent with this, *unc-101* mutations are not capable of bypassing the complete lack of either *let-23* or *let-60 ras*, suggesting that an *unc-101* mutation (and therefore the removal of one pathway of negative regulation) is not sufficient to

promote vulval fates in the absence of any inductive signal. Also, ablation of the gonad of the animals of the double mutants of *unc-101(sy108); let-23(sy1)* in their L1 larval stage revealed that the vulval differentiation of the double mutants were gonad-dependent (data not shown), suggesting that the vulval differentiation in these animals still require the signal from the gonad.

Roles of clathrin-associated complexes and *unc-101*

The function of the AP complexes is thought to couple the clathrin triskelion with distinct receptors and maybe with membranes. The core of the complex (fig. 1), which contains N terminal halves of large chains and entire region of medium chain and small chain of AP complex, can bind to the clathrin units. The function of each component of AP complex is not well understood. It has been suggested that AP50 and AP47 might have kinase activity (Pauloin et al., 1982; Campbell et al., 1984; Manfredi et al., 1987; Keen et al., 1987; Meresse et al., 1990), although sequence analysis does not show any homology to known kinase proteins. It is possible that these medium chains function as regulators of the clathrin-associated protein complexes; kinase activity would be one way of exerting a regulatory effect.

Since *unc-101* mutations have many different pleiotropic effects, the wild-type *unc-101* protein has important functions in many different types of tissues such as neurons, vulval precursor cells, and male spicule cells. How would *unc-101* function in these cells? One function is negative regulation of EGF receptor mediated signal transduction as in vulval differentiation. The involvement of *unc-101* in the *let-23* pathway may be exhibited in some subsets of cells such as vulval precursor cells, but it is also possible that *unc-101* may regulate different types of receptor pathways in different types of cells or tissues. Further genetic analysis may help reveal the full spectrum of *unc-101* interaction.

We still do not know the molecular mechanism of *unc-101* action in vulval induction pathway. However, based on the results from the biochemical studies on trans-Golgi coated vesicles and assuming that *unc-101* is a trans-Golgi clathrin-associated protein, one can predict many possible molecular mechanisms by which *unc-101* could act within the cells involved in the C. elegans vulval induction. One would be regulation of secretion of *lin-3* signal molecules within the anchor cell: *unc-101* might be involved in negative regulation of *lin-3* signal production by sequestering Lin-3 protein from the secretion route after its synthesis. However, this model seems unlikely because it is difficult to explain why *unc-101* mutants in the *let-23*(+) background do not have any excessive vulval differentiation, since overexpression of *lin-3* causes excessive vulval differentiation (Hill and Sternberg, 1992). Another possible role of *unc-101* would be regulation of transport of *let-23* receptors after their biosynthesis: *unc-101* might regulate *let-23* receptors by maintaining an intracellular pool of the receptors after their synthesis. This action could provide a post-translational regulation of the protein activity. Hence, in the absence of intracellular pool of the receptors, more *let-23* receptors that are less active could reach the cell surface, being capable of transducing more signal. A third possible role of *unc-101* would be attenuation of the signal transduction after activation of the molecules in the pathway by directing intracellular degradation: One of the best known functions of the coated vesicles on the trans-Golgi is sorting of the lysosomal enzymes. This is accomplished by sorting mannose-6-phosphate receptors, which recognize lysosomal enzymes tagged with mannose-6-phosphate, and transferring these receptor/ ligand complexes to the lysosome. *unc-101* might be indirectly involved in the down-regulation of the signal transduction components through establishing the lysosomal compartments.

For now, *unc-101* is the only case in which mutations of the clathrin AP genes cause any visible phenotype. Since *unc-101* mutations have pleiotropic phenotypes in many different tissues, and since murine AP47 and nematode *unc-101* are functionally interchangeable, one can study the function of clathrin coated vesicles in the regulation of a signal transduction processes. Further study of *unc-101* and homologs of other components of the clathrin coated vesicles on the plasma membrane and the trans-Golgi compartment will help understand the nature of the coated vesicles and their roles in the signal transduction pathway.

Experimental Procedures

Strains and genetic methods

Methods for culturing and handling the nematode and general genetic methods were described by Brenner (Brenner, 1974.) All genetic experiments were performed at 20 °C. Mutagenesis using trimethyl psoralen was described by Edgar (personal communication). The standard strain N2 was from Brenner (1974). The strains for examining interaction of *unc-101* were as follows: *lin-3(e1417), lin-3(n378), let-23(sy1), let-23(sy97), let-23(sy12), let-23(n1045), lin-2(n768), lin-2(e1309), lin-7(e1413), lin-10(e1439), sem-5(n2019), let-60(sy100dn), let-60(n2021), lin-45(sy96).* The starting strain for identifying the polymorphisms associated with *unc-101*, MT3618 (*unc-75 (e950) ced-1 (n1506) unc-59 (e261)* , which has a portion of chromosome I (between *unc-75* and *unc-59*) from the mutator TR679 strain, was kindly provided by S. Glass. The *unc-101* alleles sequenced for localization of mutations were *m1* (D. Riddle), *rh6* (E. Hedgecock), *sy108*, *sy161* (Jongeward and Sternberg, in prep), *sy168*, *sy169*, *sy241* (in this study), and *sy237* (J. Lee, unpublished result).

Inverse PCR and mapping of the polymorphisms

The method for inverse PCR was described by Ochman et al. (1988). We picked recombinants from heterozygous progeny of + + *sy108* + / *unc-75 ced-1* + *unc-59*, and made them homozygous. We digested genomic DNAs of these recombinants with HindIII, diluted, and self-ligated them, then used them as templates for PCR with Tc1 internal primers of Hill and Sternberg (1992). After finding two polymorphisms, TCUNC101A and TCUNC101E, 1.1kb and 0.8kb long, respectively, on an analytic agarose gel, we subcloned the fragments into a pBluescript vector. We used the inserts of these subclones as a probe for physical and genetic mapping (Williams et al., 1992). We determined the DNA sequence of TCUNC101E polymorphism and made two PCR primers for further mapping.

EPR1: 5' GGTGA TAGCA CCATA TGGTT CC 3'

EPR2: 5' ATATA GTGCT GTGCG GAACT C 3'

We designed these primers so that if the recombinants had the polymorphic transposon, the PCR-amplified band using either of these primers and the Tc1 internal primer would be 125 bp long, and if not, an 80 bp band would be amplified from EPR1 and EPR2 as extending primers.

Cosmids and C. elegans physical map

We obtained all cosmids and the physical map data from A. Coulson and J. Sulston (MRC, Cambridge, UK; Coulson et al., 1988, Coulson et al., 1986).

DNA -mediated transformation of *unc-101* mutants

Microinjection of cosmids or subcloned DNAs was described by Mello et al. 1991. We used *unc-101 (sy108)* animals as host strain for rescuing the Unc and the

lethality of the visible allele. We co-injected *unc-101 (sy108)* animals with pRF4, which bears a dominant *rol*-6 mutation. Selection of this marker phenotype facilitates selecting the transgenic animals. We used *unc-101(sy108)*; *let-23 (sy1)* animals for examining the rescue of the suppression of the *let-23 (sy1)* vulvaless phenotype. We injected these animals with the rescuing subclone pJL5 without any other marker, because the rescue of the Unc phenotype itself serves as a good marker for the presence of the transgene. We used *sy216 / hIn1 unc-54 (h1040)* heterozygotes for examining the rescue of the lethality of *sy216*. *hIn1* is an inversion that suppresses recombination in the region between *unc-75* and *unc-59*. We co-injected the W05A3 cosmid and the pRF4 marker DNAs , picked the rolling F1 transgenic animals , and transferred twenty F2 individual animals to new plates and checked their segregation of *unc-54* marker. All of them segregated *unc-54,* indicating that there was no animal of genotype *sy216/ sy216; [Ex W05A3 + rol-6]* .

Genomic DNA and cDNA manipulations and Sequencing

All procedures of handling genomic DNA and cDNA were described (Sambrook et al., 1989).

Sequence data analysis

Macvector software package of IBI (New Haven CT.) and the software of the Genetics Computer Group v7.0 (Deverreux et al., 1984; GCG, 1991) was used to edit the genomic and cDNA sequences. Database search was performed using the BLAST program. Pileup and Prettyplot commands were used to generate the comparison of the amino acid sequences.

Construction of hybrid plasmid with *unc-101* and AP47

We digested the rescuing plasmid pJL2 with NruI and EcoRV, and purified the 0.9kb fragment from a low melting point agarose gel and ligated it with the NruI/ EcoRV fragment from the *unc-101* cDNA subclone. The resulting plasmid was the *unc-101* cDNA hybrid gene. We made two PCR primers from the AP47 cDNA sequence to amplify the corresponding region of NruI/ EcoRV fragment of *unc-101* cDNA.

47-1: 5' CGACA ACTTT GTCAT CATCT A;

47-2 : 5' ATCCA CTCTT CTCAA TGATT TTC 3'

To facilitate subcloning, we replaced three most 5' nucleotides of 47-1 and 47-2 primers with the recognition sequence of NruI and EcoRV, respectively. This replacement does not change the coding amino acid. We ligated the amplified DNA into the pJL2 NruI/ EcoRV fragment. The correct reading frame was confirmed by regeneration of the NruI and EcoRV sites. This construct has the 5' region of *unc-101* including the 5' region of the rescuing plasmid pJL2 and 5' coding region with two introns up to cDNA nucleotide 388, fused in frame to the AP47 cDNA from 389 to 1281, *unc-101* cDNA nucleotide from 1282 to the stop codon, and all the 3' untranslated region of *unc-101*.

Determination of mutations

We made ten PCR primers from the intron sequences, 5' end and 3' nontranslated region as follows.

INT1: TTCCG CTAAT TTTCT CCGG

INT2: ATTGC GTCAT TTTCA ACGG

INT3: CGCTC CAATG ATAAA ACAC

INT4: GCATT TTCGC ATTGG AGCG

INT5: AAATG TGTTT TTCGA CTCG

INT6: AAAAA CTAGG CCACA TCAC

INT7: AAGTC AGGCC ATGCC TCAA

INT8: CATAA ATCTC ACATT GGGCA

INT9: GAGAA TTATG TGATT TTTTG

INT10: CTCGG CCACG GTCGT TTTT

With these PCR primers, we could amplify all the exons and their flanking intron sequences. We used genomic DNA or single worms of the homozygous mutant in PCR to amplify the exons and their boundaries. We directly sequenced the amplified DNAs using the same sets of primers (Kretz et al., 1989). To amplify genomic DNA from the *sy216* dead animals, we picked two or three arrested animals, and performed PCR as described above. As an internal positive control, we used a set of *let-23* PCR primers in the same PCR reaction tube with *unc-101* primers. We tried five different sets of *unc-101* primers, but only recovered an amplified band of *let-23* genomic DNA from the *let-23* primers.

Figure 1. Clathrin vesicles and their associated protein complexes

a. coated pits and vesicles.

A coated pit is composed of membrane fraction, collected membrane proteins, and clathrin triskelion cage with its associated protein complex. Coated pits are invaginated to form coated vesicles, which travel to their destinations such as lysosome and the plasma membrane (Modified from Pearse and Robinson 1990).

b. Schematic structure of the clathrin associated protein complex.

AP-1 complex is composed of four different peptide chains: two large chains, β, γ, one medium chain AP47, and one small chain AP19. AP-2, not shown in the figure, has similar structure to that of AP-1, and is composed of two large chains, α, β, one medium chain AP50, and one small chain AP17 (Modified from Pearse and Robinson 1990).

Figure 1-a

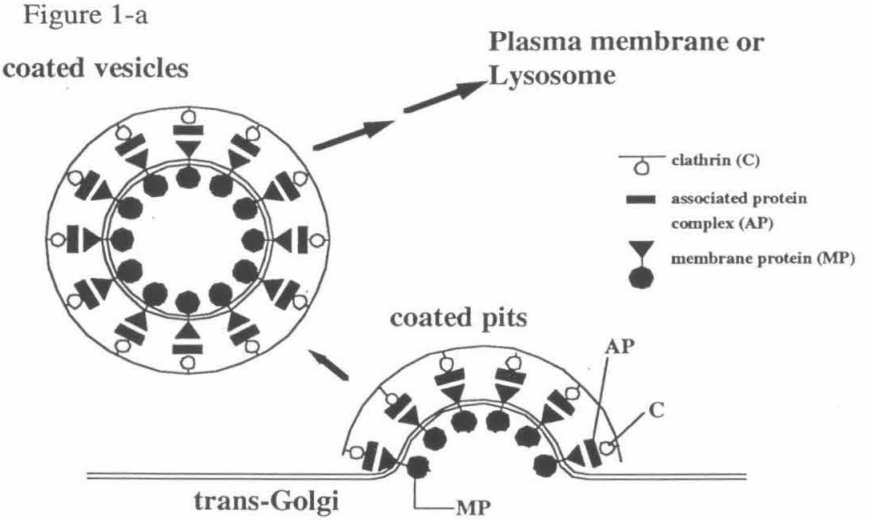


Figure 1-b

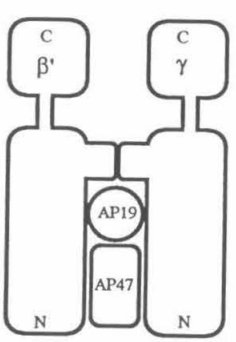
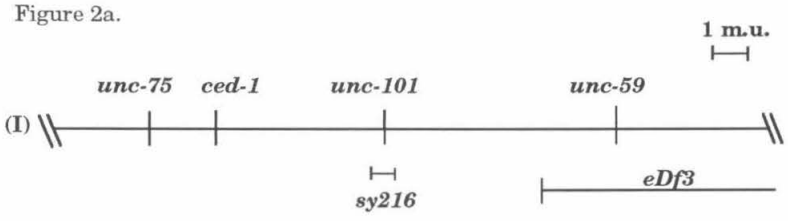


Figure 2. Isolation of null alleles of *unc-101.*

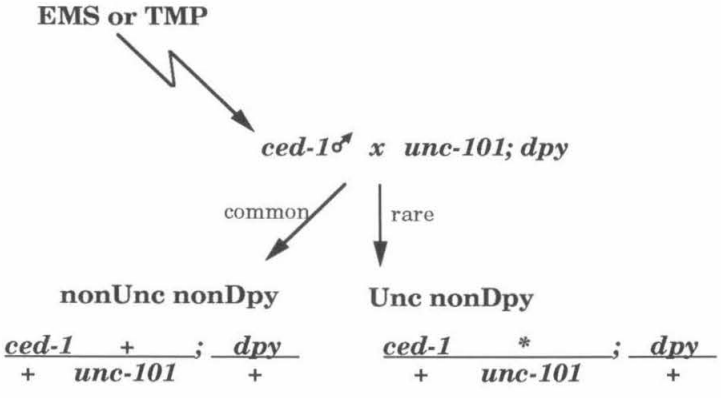
a. genetic map around *unc-101.* The genetic distances are based on the mapping data from other labs and this study.

b, c. Non-complementation screens for the isolation of the null alleles of *unc-101.* EMS or trimethylpsoralen was used as mutagen. b. We mutagenized *ced-1* males, and mated them with *unc-101; dpy* strain where *dpy* is a marker for distinguishing the cross progeny from the self progeny. Self progeny should be dumpy (Dpy), uncoordinated (Unc), and most of the cross progeny should be non-dumpy (nDpy), non-uncoordinated (nUnc). We looked for non-dumpy (nDpy), uncoordinated (Unc) F1 animals whose *ced-1* chromosome should have a new allele of *unc-101. eDf24* is a deficiency that deletes the rRNA genes on the chromosome I, conferring lethality to the homozygotes for this deficiency. *hIn1* is an inversion of chromosome I that suppresses recombination in the region between *unc-75* and *unc-54.* This balancer is marked with *unc-54,* facilitating the discrimination of the homozygotes. After mutagenizing the heterozygotes of *unc-101(sy108); = eDf24 / hIn1[+ unc-54(h1040) +],* we looked for the Unc-101 animals whose *hIn1* chromosome should have a new allele of *unc-101.*

Figure 2a.



2b.



2c.

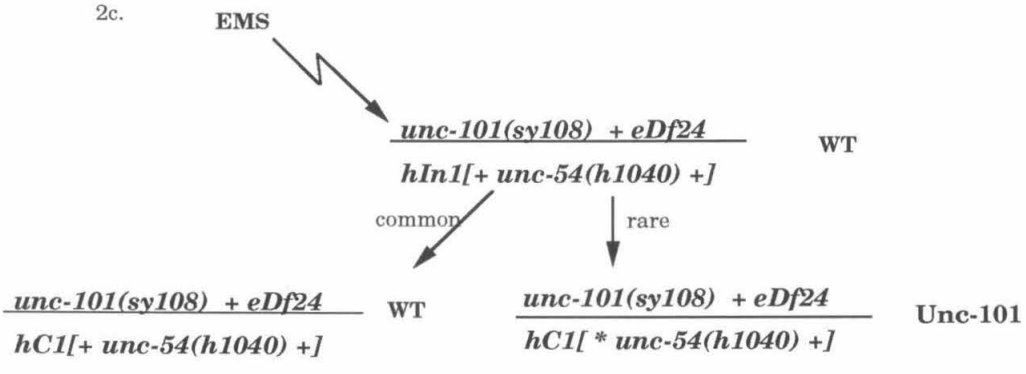


Figure 3. unc-101 is a negative regulator of vulval induction pathway.

unc-101 mutations strongly suppress mutations of *lin-2, lin-7, lin-10,* and *let-23*, suggesting that *unc-101* may act at or near these genes (see text and table 1 for details).

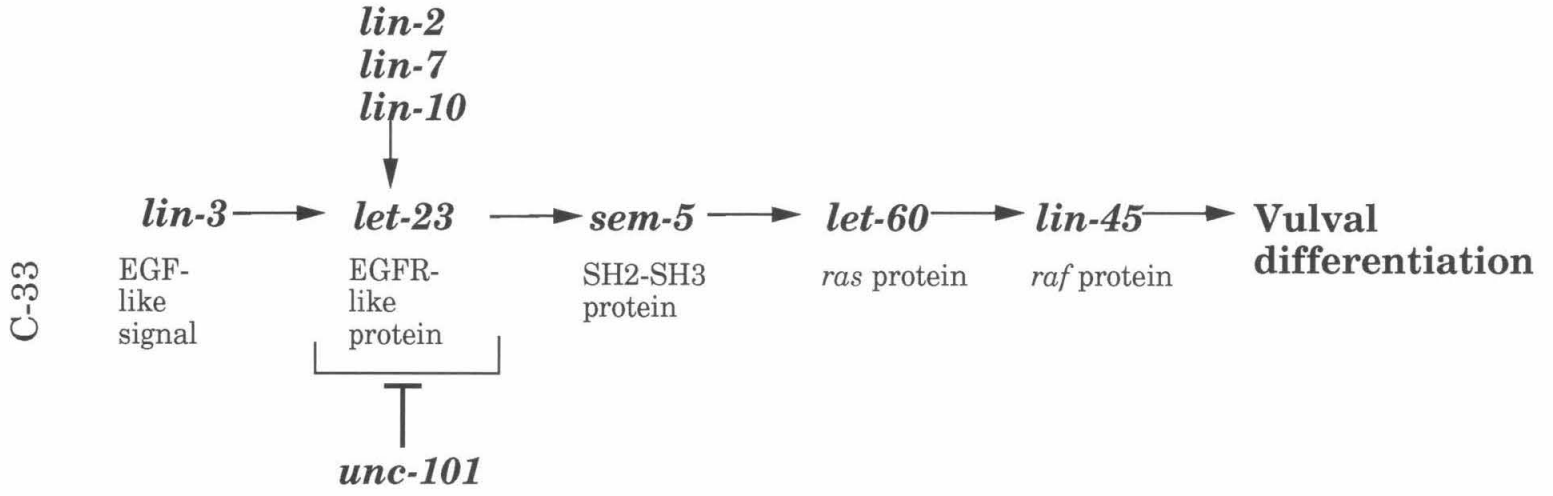

lin-2
lin-7
lin-10
lin-3
let-23
sem-5
let-60
lin-45
Vulval differentiation
EGF-like signal
EGFR-like protein
SH2-SH3 protein
ras protein
raf protein
unc-101
C-33

Figure 3

Figure 4. Cloning of unc-101 gene

a. Genetic and physical map near *unc-101*. The genetic distance between *ced-1* and *unc-101* is about 5 map units. The two RFLPs are marked as triangles. TCUNC101A is in the same YAC as the *ced-1* YAC. TCUNC101E is 600kb from TCUNC101A, and genetically inseparable from *unc-101(+)* marker. The W05A3 cosmid, but not the other 4 cosmids, had the ability to rescue Unc phenotype of *unc-101(sy108)*.

b. Map of W05A3 cosmid and its subclones. pJL5, a 6.3 kb subclone, is the smallest genomic region capable of rescuing. pJL3 does not rescue the phenotype, suggesting that this restriction enzyme site disrupts the *unc-101* gene. Restriction sites shown are as follows: S, SpeI; N, NdeI; H, HindIII; P, PstI; and A, ApaI.

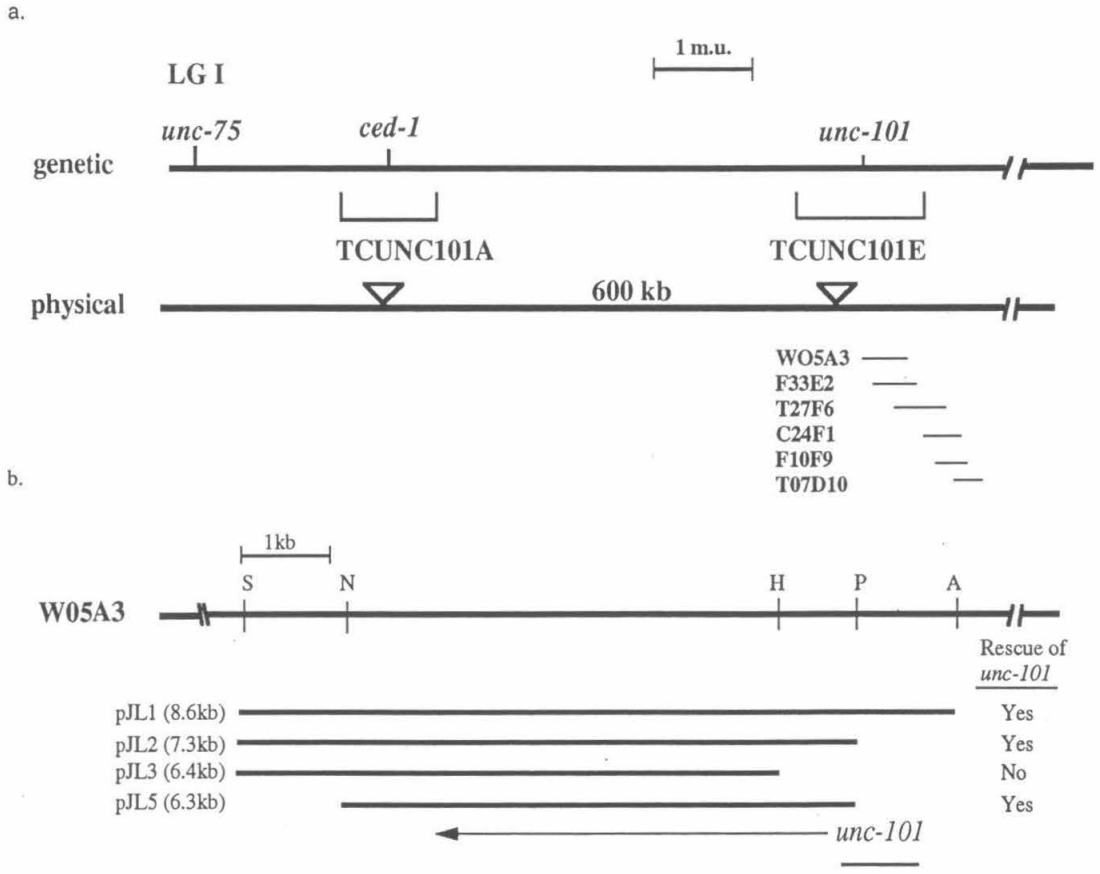
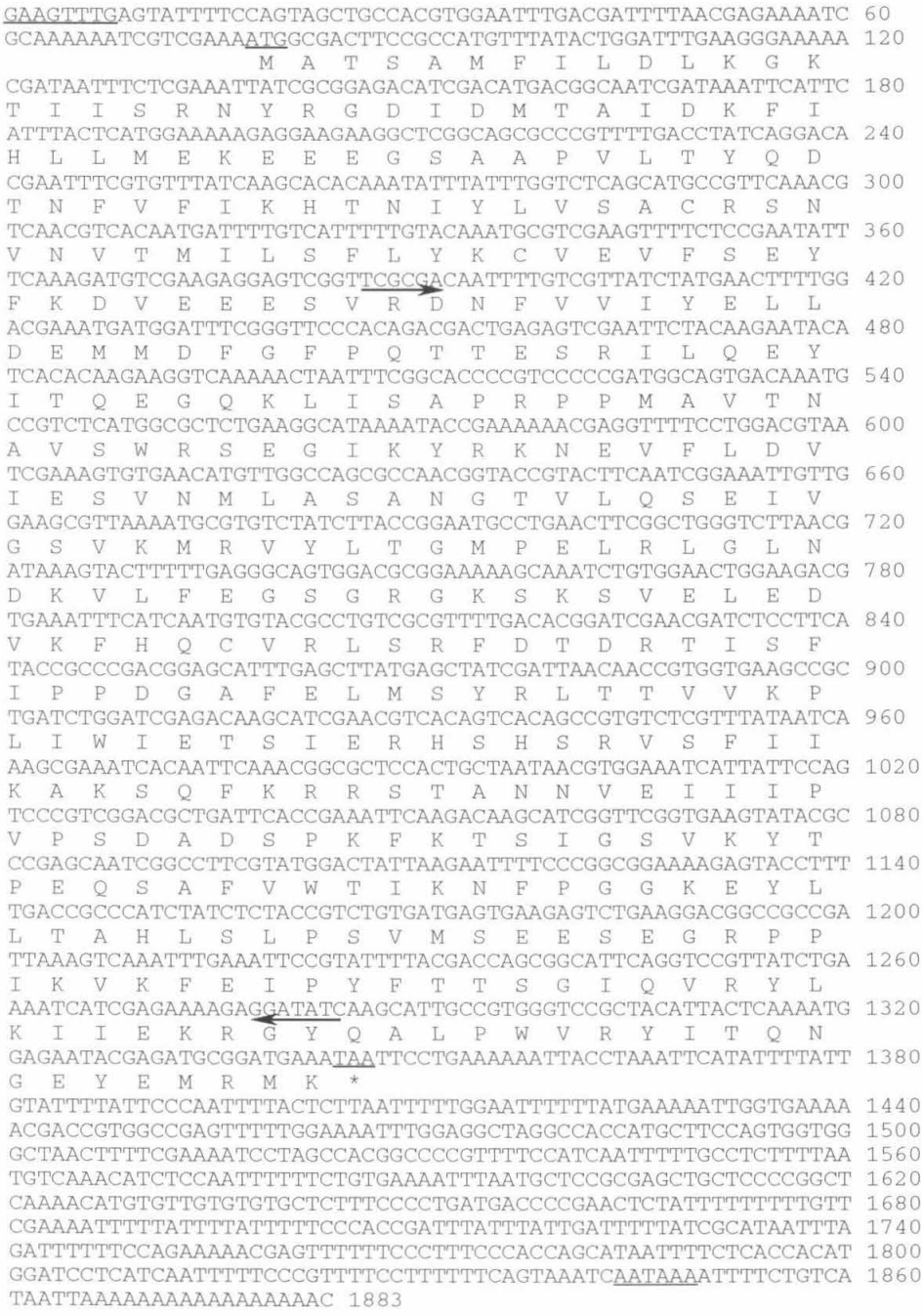

a.
LG I
1 m.u.
unc-75
ced-1
unc-101
genetic
TCUNC101A
TCUNC101E
physical
600 kb
WO5A3
F33E2
T27F6
C24F1
F10F9
T07D10
b.
1kb
S
N
H
P
A
W05A3
Rescue of unc-101
pJL1 (8.6kb)
Yes
pJL2 (7.3kb)
Yes
pJL3 (6.4kb)
No
pJL5 (6.3kb)
Yes
unc-101

Figure 5. Genomic structure and cDNA sequence of *unc-101.*

a. *unc-101* cDNA sequence and its predicted translation product. The region identical to the 3' end of SL-1 sequence is underlined. The start and stop codons, and a poly adenylation signal sequence are also underlined. The sites for the construction of the mouse AP47 hybrid gene are marked as lines with an arrowhead.

b. Genomic structure of *unc-101* is shown. *unc-101* has seven exons and six introns. The size of introns varies from 50 bp to 1.0 kb. The numbers above the structures represent the last nucleotide of exons. The locations and characteristics of mutations are marked as such.

Figure 5a


GAAGTTTGAGTATTTTCCAGTAGCTGCCACGTGGAATTTGACGATTTTAACGAGAAAATC 60
GCAAAAAATCGTCGAAAATGGCGACTTCCGCCATGTTTATACTGGATTTGAAGGGAAAAA 120
M A T S A M F I L D L K G K
CGATAATTTCTCGAAATTATCGCGGAGACATCGACATGACGGCAATCGATAAATTCATTC 180
T I I S R N Y R G D I D M T A I D K F I
ATTTACTCATGGAAAAAGAGGAAGAAGGCTCGGCAGCGCCCGTTTTGACCTATCAGGACA 240
H L L M E K E E E G S A A P V L T Y Q D
CGAATTTCGTGTTTATCAAGCACACAAATATTTATTTGGTCTCAGCATGCCGTTCAAACG 300
T N F V F I K H T N I Y L V S A C R S N
TCAACGTCACAATGATTTTGTCATTTTTGTACAAATGCGTCGAAGTTTTCTCCGAATATT 360
V N V T M I L S F L Y K C V E V F S E Y
TCAAAGATGTCGAAGAGGAGTCGGTTCGCGACAATTTTGTCGTTATCTATGAACTTTTGG 420
F K D V E E E S V R D N F V V I Y E L L
ACGAAATGATGGATTTCGGGTTCCCACAGACGACTGAGAGTCGAATTCTACAAGAATACA 480
D E M M D F G F P Q T T E S R I L Q E Y
TCACACAAGAAGGTCAAAAACTAATTTCGGCACCCCGTCCCCCGATGGCAGTGACAAATG 540
I T Q E G Q K L I S A P R P P M A V T N
CCGTCTCATGGCGCTCTGAAGGCATAAAATACCGAAAAAACGAGGTTTTCCTGGACGTAA 600
A V S W R S E G I K Y R K N E V F L D V
TCGAAAGTGTGAACATGTTGGCCAGCGCCAACGGTACCGTACTTCAATCGGAAATTGTTG 660
I E S V N M L A S A N G T V L Q S E I V
GAAGCGTTAAAATGCGTGTCTATCTTACCGGAATGCCTGAACTTCGGCTGGGTCTTAACG 720
G S V K M R V Y L T G M P E L R L G L N
ATAAAGTACTTTTTGAGGGCAGTGGACGCGGAAAAAGCAAATCTGTGGAACTGGAAGACG 780
D K V L F E G S G R G K S K S V E L E D
TGAAATTTCATCAATGTGTACGCCTGTCGCGTTTTGACACGGATCGAACGATCTCCTTCA 840
V K F H Q C V R L S R F D T D R T I S F
TACCGCCCGACGGAGCATTTGAGCTTATGAGCTATCGATTAACAACCGTGGTGAAGCCGC 900
I P P D G A F E L M S Y R L T T V V K P
TGATCTGGATCGAGACAAGCATCGAACGTCACAGTCACAGCCGTGTCTCGTTTATAATCA 960
L I W I E T S I E R H S H S R V S F I I
AAGCGAAATCACAATTCAAACGGCGCTCCACTGCTAATAACGTGGAAATCATTATTCCAG 1020
K A K S Q F K R R S T A N N V E I I I P
TCCCGTCGGACGCTGATTCACCGAAATTCAAGACAAGCATCGGTTCGGTGAAGTATACGC 1080
V P S D A D S P K F K T S I G S V K Y T
CCGAGCAATCGGCCTTCGTATGGACTATTAAGAATTTTCCCGGCGGAAAAGAGTACCTTT 1140
P E Q S A F V W T I K N F P G G K E Y L
TGACCGCCCATCTATCTCTACCGTCTGTGATGAGTGAAGAGTCTGAAGGACGGCCGCCGA 1200
L T A H L S L P S V M S E E S E G R P P
TTAAAGTCAAATTTGAAATTCCGTATTTTACGACCAGCGGCATTCAGGTCCGTTATCTGA 1260
I K V K F E I P Y F T T S G I Q V R Y L
AAATCATCGAGAAAAGAGGATATCAAGCATTGCCGTGGGTCCGCTACATTACTCAAAATG 1320
K I I E K R G Y Q A L P W V R Y I T Q N
GAGAATACGAGATGCGGATGAAATAATTCCTGAAAAAATTACCTAAATTCATATTTTATT 1380
G E Y E M R M K *
GTATTTTATTCCCAATTTTACTCTTAATTTTTGGAATTTTTTATGAAAAATTGGTGAAAA 1440
ACGACCGTGGCCGAGTTTTTGGAAAATTTGGAGGCTAGGCCACCATGCTTCCAGTGGTGG 1500
GCTAACTTTTCGAAAATCCTAGCCACGGCCCCGTTTTCCATCAATTTTTGCCTCTTTTAA 1560
TGTCAAACATCTCCAATTTTTTTCTGTGAAAATTTAATGCTCCGCGAGCTGCTCCCCGGCT 1620
CAAAACATGTGTTGTGTGTGCTCTTTCCCCTGATGACCCCGAACTCTATTTTTTTTTTGTT 1680
CGAAAATTTTTATTTTATTTTTCCCACCGATTTATTTATTGATTTTTATCGCATAATTTA 1740
GATTTTTTCCAGAAAAACGAGTTTTTTCCCTTTCCCACCAGCATAATTTTCTCACCACAT 1800
GGATCCTCATCAATTTTTCCCGTTTTCCTTTTTTCAGTAAATCAATAAAATTTTCTGTCA 1860
TAATTAAAAAAAAAAAAAAAAC 1883

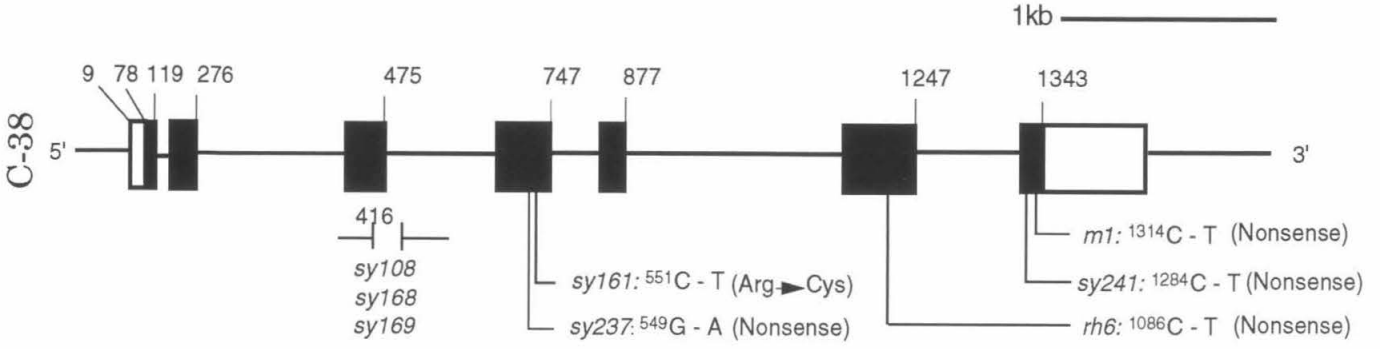

1kb
C-38
5'
3'
9
78
119
276
475
747
877
1247
1343
416
sy108
sy168
sy169
sy161: 551C - T (Arg→Cys)
sy237: 549G - A (Nonsense)
rh6: 1086C - T (Nonsense)
sy241: 1284C - T (Nonsense)
m1: 1314C - T (Nonsense)

Figure 5b

Figure 6. AP50 homolog in C. elegans: cDNA sequence of an AP50 homolog in C. elegans and its predicted translation product. The start and stop codons, and a putative polyadenylation signal are underlined.

```
CGGTTTGAGCAAACTCGGGGATGATTGGTGGATTGTTCGTTTACAATCACAAAGGAGAAG 60
                      M  I  G  G  L  F  V  Y  N  H  K  G  E
TGCTCATTTCGAGAATCTATCGAGACGATGTAACCCGGAACGCAGTCGACGCCTTCCGAG 120
V  L  I  S  R  I  Y  R  D  D  V  T  R  N  A  V  D  A  F  R
TCAACGTCATCCATGCCCGACAGCAAGTTCGCTCGCCAGTCACCAACATGGCTCGTACTT 180
V  N  V  I  H  A  R  Q  Q  V  R  S  P  V  T  N  M  A  R  T
CGTTCTTCCATGTGAAGCGTGGCAACGTCTGGATTTGTGCGGTGACACGTCAAAATGTCA 240
S  F  F  H  V  K  R  G  N  V  W  I  C  A  V  T  R  Q  N  V
ACGCTGCCATGGTTTTTGCGTTCTTGAAACGCTTCGCCGACACCATGCAGTCTTACTTTG 300
N  A  A  M  V  F  A  F  L  K  R  F  A  D  T  M  Q  S  Y  F
GAAAACTGAACGAGGAGAATGTGAAGAACAACTTTGTGTTGATTTATGAGTTGCTCGACG 360
G  K  L  N  E  E  N  V  K  N  N  F  V  L  I  Y  E  L  L  D
AGATTCTCGACTTTGGATACCCCCAGAATACGGACCCTGGTGTGCTGAAAACTTTCATCA 420
E  I  L  D  F  G  Y  P  Q  N  T  D  P  G  V  L  K  T  F  I
CCCAGCAAGGAGTCAGAACAGCTGATGCTCCTGTCCCAGTGACCAAAGAGGAGCAGTCAC 480
T  Q  Q  G  V  R  T  A  D  A  P  V  P  V  T  K  E  E  Q  S
AAATCACGTCTCAAGTGACTGGCCAAATTGGATGGCGTCGGGAGGGTATTAAGTACCGCC 540
Q  I  T  S  Q  V  T  G  Q  I  G  W  R  R  E  G  I  K  Y  R
GAAATGAGCTCTTCCTGGATGTTATTGAATATGTCAACTTGCTCATGAATCAACAAGGAC 600
R  N  E  L  F  L  D  V  I  E  Y  V  N  L  L  M  N  Q  Q  G
AAGTATTATCTGCTCATGTTGCCGGAAAAGTTGCGATGAAATCCTATTTGAGTGGAATGC 660
Q  V  L  S  A  H  V  A  G  K  V  A  M  K  S  Y  L  S  G  M
CGGAGTGCAAATTTGGCATCAACGACAAAATTACCATCGAAGGAAAGTCGAAGCCAGGAA 720
P  E  C  K  F  G  I  N  D  K  I  T  I  E  G  K  S  K  P  G
GTGATGATCCAAACAAAGCAAGCCGTGCCGCAGTGGCCATTGATGACTGTCAATTCCACC 780
S  D  D  P  N  K  A  S  R  A  A  V  A  I  D  D  C  Q  F  H
AATGCGTGAAGCTGACGAAATTTGAGACGGAGCACGCGATTTCTTTTATCCCACCGGACG 840
Q  C  V  K  L  T  K  F  E  T  E  H  A  I  S  F  I  P  P  D
GCGAGTACGAGCTGATGAGATACCGTACCACTAAGGATATCCAACTGCCATTCCGTGTGA 900
G  E  Y  E  L  M  R  Y  R  T  T  K  D  I  Q  L  P  F  R  V
TCCCATTGGTTCGTGAAGTGTCTCGTAACAAGATGGAAGTTAAGGTTGTCGTCAAGTCTA 960
I  P  L  V  R  E  V  S  R  N  K  M  E  V  K  V  V  V  K  S
ACTTCAAGCCATCCCTTCTTGCTCAAAAGCTCGAAGTTCGCATTCCAACCCCACCAAATA 1020
N  F  K  P  S  L  L  A  Q  K  L  E  V  R  I  P  T  P  P  N
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TCGATCTTCTCTCAACTGGAAACGTTGAGAAGAAGAAATGGAATCGCCCACCGGTCAGCA 1200
I  D  L  L  S  T  G  N  V  E  K  K  K  W  N  R  P  P  V  S
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M  N  F  E  V  P  F  A  P  S  G  L  K  V  R  Y  L  K  V  F
AGCCAAAACTGAACTATTCGGATCATGACGTCATCAAATGGGTTCGTTACATTGGAAGAT 1320
E  P  K  L  N  Y  S  D  H  D  V  I  K  W  V  R  Y  I  G  R
CGGGACTGTATGAAACCAGATGCTAGAACTTCACCCAACCCTTTCTATCTTCATTTGCTC 1380
S  G  L  Y  E  T  R  C  *
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TTGCTTCATACAATTTTTAATTTTTTGTAATATTTTTGTATTATTTTCCAAATTTCATAA 1500
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CAAAATTCAAAACCCCCGCCCCACCCCGTTCCATCTTTATTTATTGTCAGTGTAAAATTT 1680
CAAAATTTCTGGAATCTTTTTCCATCATAAAATTCACTTCAAACGTCTTCTGACCCGTTG 1740
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TCAAAATTTTCAAATTTCTATTGTTCATTTCTCATTTCTAGGTCGCAATTCAATAAGTTT 1920
GTAATTCAAATGAATAGCATAATATTTATTTTAAATAAAAGTTTTTTTTTATTAAAGCGGA 1980
ATTCC 1985
```

Figure 7. unc-101 is a clathrin-associated protein.

a. Amino acid sequence alignment of homologs of *unc-101*. AP47 and AP50 are the medium chains of trans-Golgi clathrin-associated protein complex and plasma membrane clathrin-associated protein complex, respectively (Nakayama et al., 1991; Thurieau et al., 1988). CEAP50 is an AP50 homolog in C. elegans (this study). This alignment only highlights the residues that are identical between Unc-101 and AP47 or between all four homologs.

b. Diagram of the comparison of identity among the homologs of medium chains of clathrin-associated complexes. The numbers represent the identity at amino acid sequence level. The most prominent homologies are marked by bold lines. This shows that *unc-101* and AP47 are of a group, and AP50 and CEAP50 are of another.

```
UNC-101   MATSAMFILD LKGKTIISRN YRGDIDMTAI DKFIHLLMEK EEEGSAAPVL
   AP47   MSASAVYVLD LKGKVLICRN YRGDVDMSEV EHFMPILMEK EEEGMLSPIL
 CEAP50   .MIGGLFVYN HKGEVLISRI YRDDVTRNAV DAFRVNVIHA RQQVR.SPVT
   AP50   .MIGGLFIYN HKGEVLISRV YRDDIGRNAV DAFRVNVIHA RQQVR.SPVT

UNC-101   TYQDTNFVFI KHTNIYLVSA CRSNVNVTMI LSFLYKCVEV FSEYFKDVEE
   AP47   AHGGVRFMWI KNNNLYLVAT SKKNACVSLV FSFLYKVVQV FSEYFKELEE
 CEAP50   NMARTSFFHV KRGNVWICAV TRQNVNAAMV FAFLKRFADT MQSYFGKLNE
   AP50   NIARTSFFHV KRSNIWLAAV TKQNVNAAMV FEFLYKMCDV MAAYFGKISE

UNC-101   ESVRDNFVVI YELLDEMMDF GFPQTTESRI LQEYITQEGQ K.........L
   AP47   ESIRDNFVII YELLDELMDF GYPQTTDSKI LQEYITQEGH K.........L
 CEAP50   ENVKNNFVLI YELLDEILDF GYPQNTDPGV LKTFITQQGV RTADAPVPVT
   AP50   ENIKNNFVLI YELLDEILDF GYPQNSETGA LKTFITQQGI KSQHQ....T

UNC-101   .ISAPRPPMA VTNAVSWRSE GIKYRKNEVF LDVIESVNML ASANGTVLQS
   AP47   ETGAPRPPAT VTNAVSWRSE GIKYRKNEVF LDVIEAVNLL VSANGNVLRS
 CEAP50   KEEQSQITSQ VTGQIGWRRE GIKYRRNELF LDVIEYVNLL MNQQGQVLSA
   AP50   KEEQSQITSQ VTGQIGWRRE GIKYRRNELF LDVLESVNLL MSPQGQVLSA

UNC-101   EIVGSVKMRV YLTGMPELRL GLNDKVLFEG SGRGKSK...  .....SVELE
   AP47   EIVGSIKMRV FLSGMPELRL GLNDKVLFDN TGRGKSK...  .....SVELE
 CEAP50   HVAGKVAMKS YLSGMPECKF GINDKITIEG KSKPGSDDPN KASRAAVAID
   AP50   HVSGRVVMKS YLSGMPECKF GMNDKIVIEK QGKGTADETS KSGKQSIAID

UNC-101   DVKFHQCVRL SRFDTDRTIS FIPPDGAFEL MSYRLTTVVK PLIWIETSIE
   AP47   DVKFHQCVRL SRFENDRTIS FIPPDGEFEL MSYRLNTHVK PLIWIESVIE
 CEAP50   DCQFHQCVKL TKFETEHAIS FIPPDGEYEL MRYRTTKDIQ LPFRVIPLVR
   AP50   DCTFHQCVRL SKFDSERSIS FIPPDGEFEL MRYRTTKDII LPFRVIPLVR

UNC-101   RHSHSRVSFI IKAKSQFKRR STANNVEIII PVPSDADSPK FKTSIGSVKY
   AP47   KHSHSRIEYM VKAKSQFKRR STANNVEIHI PVPNDADSPK FKTTVGSVKW
 CEAP50   EVSRNKMEVK VVVKSNFKPS LLAQKLEVRI PTPPNTSGVQ LICMKGKAKY
   AP50   EVGRTKLEVK VVIKSNFKPS LLAQKIEVRI PTPLNTSGVQ VICMKGKAKY

UNC-101   TPEQSAFVWT IKNFPGGKEY LLTAHLSLPS VMSEESE..G RPPIKVKFEI
   AP47   VPENSEIVWS VKSFPGGKEY LMRAHFGLPS VEAEDKE..G KPPISVKFEI
 CEAP50   KAGENAIVWK IKRMAGMKES QISAEIDLLS TGNVEKKKWN RPPVSMNFEV
   AP50   KASENAIVWK IKRMAGMKES QISAEIELLP TN..DKKKWA RPPISMNFEV

UNC-101   PYFTTSGIQV RYLKIIEK..  ..RGYQALPW VRYITQNGEY EMRMK*
   AP47   PYFTTSGIQV RYLKIIEK..  ..SGYQALPW VRYITQNGDY QLRTQ*
 CEAP50   P.FAPSGLKV RYLKVFEPKL NYSDHDVIKW VRYIGRSGLY ETRC*.
   AP50   P.FAPSGLKV RYLKVFEPKL NYSDHDVIKW VRYIGRSGIY ETRC*..
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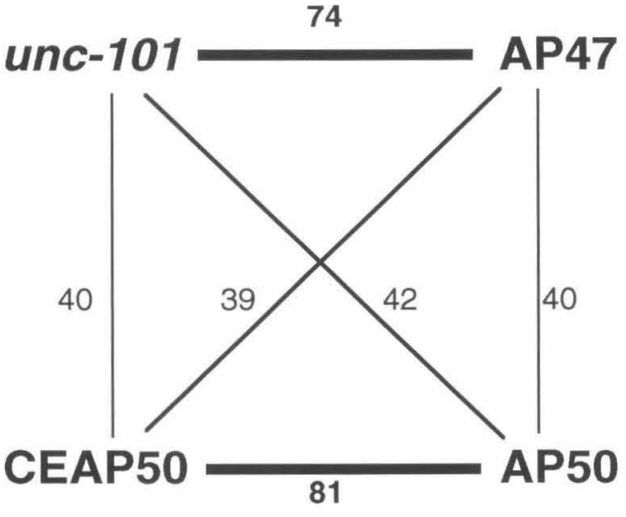


Figure 7b

Figure 8. AP47 and unc-101 are functionally equivalent.

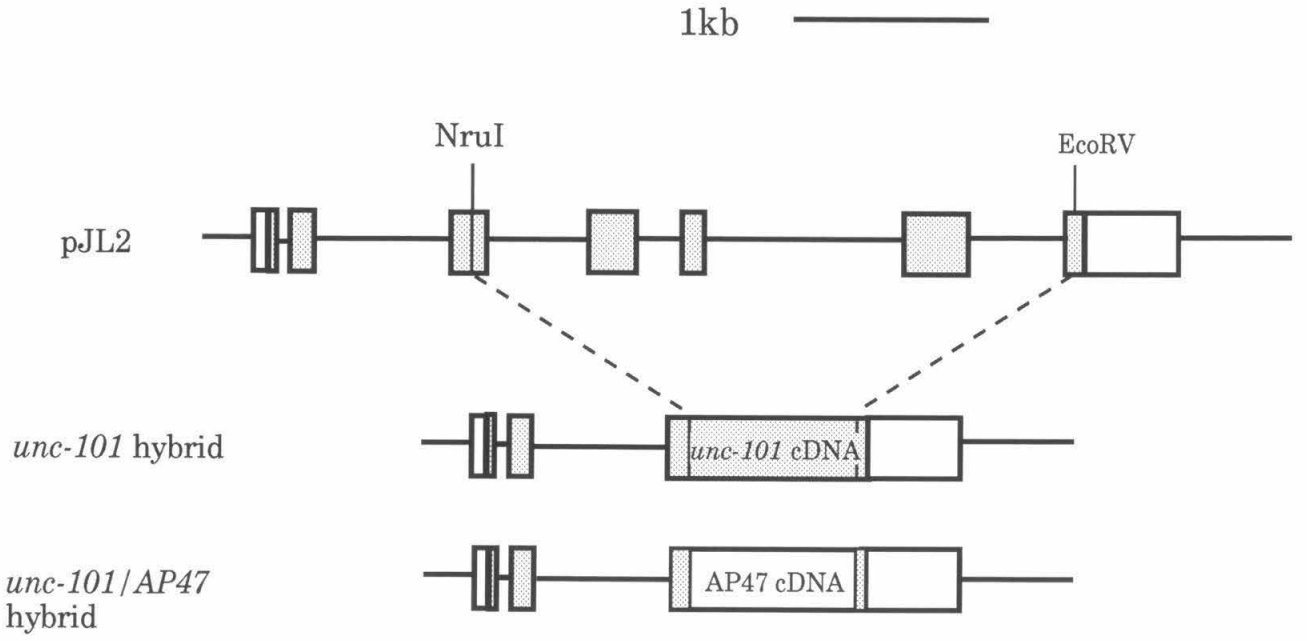
a. A schematic of hybrid construction.

The NruI/ EcoRV fragment of the rescuing plasmid pJL2 was replaced with the corresponding cDNA fragments from *unc-101* cDNA clone or mouse AP47 cDNA clone in *unc-101* hybrid gene and in *unc-101/AP47* hybrid gene, respectively.

b. Rescue of Unc phenotype. Five animals were transferred to new plates, and after given time, the tracts that the animals created by moving on the bacterial lawn were photographed. A, N2 wild type, 5 minutes after transfer; B, *unc-101 (sy108),* 40 minutes; C, transgenic animal of *unc-101 (sy108)*; Ex[AP47 hybrid], 5 minutes after transfer. The uncoordinated movement of *unc-101 (sy108)* is suppressed by the transgene.

c. Rescue of suppression of Vul phenotype of *let-23(sy1).*

The VPCs in L3 molt stage, when the VPCs should have divided twice to generate four progeny, are shown. The triangles represent the anchor cell. A, N2, three VPCs each generated four progeny ; B, *let-23(sy1)*, no VPC divided to generate four progeny; C, *unc-101(sy108)*, same as N2; D, *unc-101(sy108); let-23(sy1),* four VPCs divided to generate four progeny; E, *unc-101(sy108); let-23(sy1); Ex[AP47 hybrid]*, no VPC divided to generate four progeny.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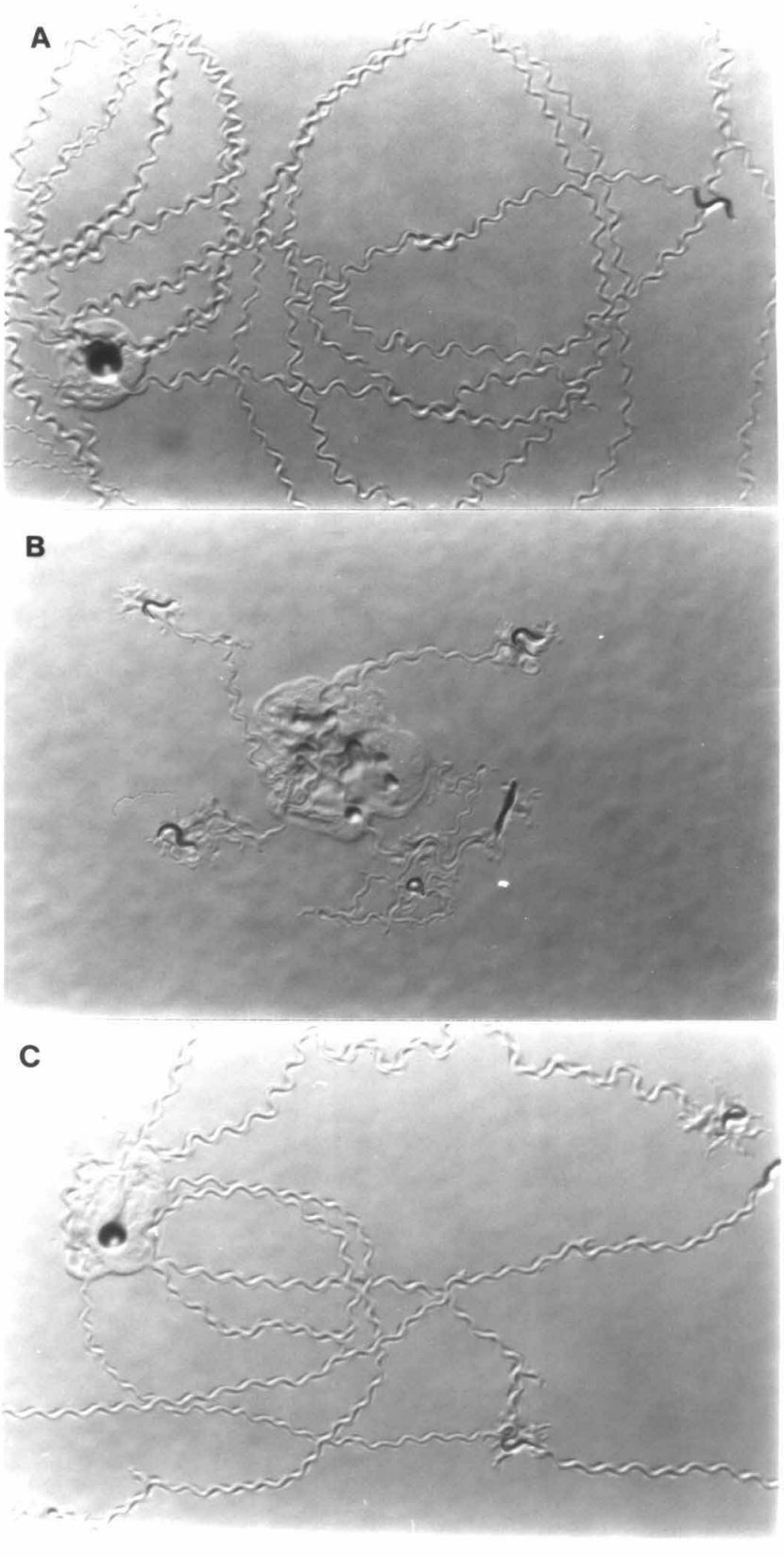
NruI
EcoRV
pJL2
unc-101 hybrid
unc-101 cDNA
unc-101/AP47
hybrid
AP47 cDNA

C-45


A
B
C

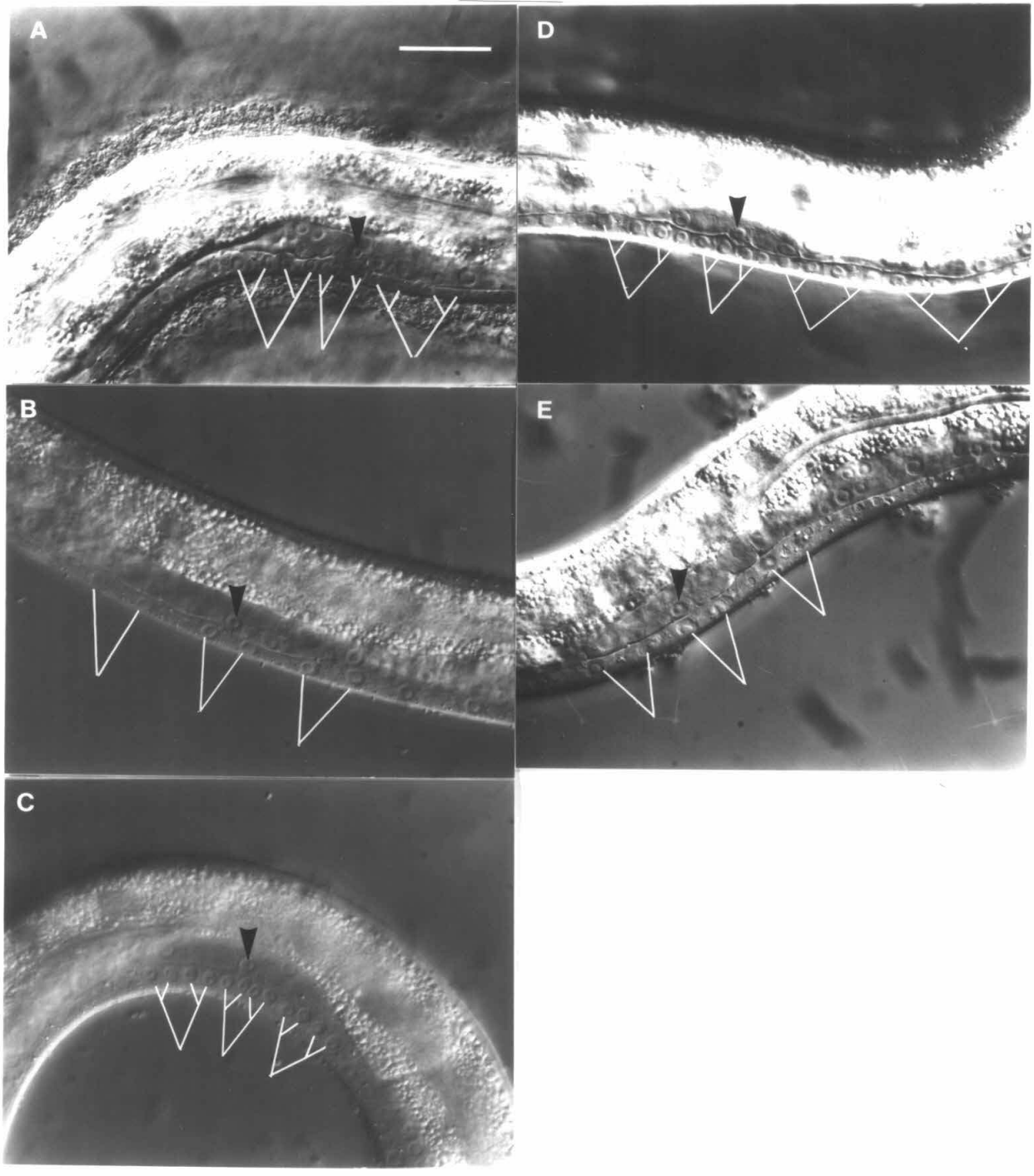

A
D
B
E
C

Table 1. Interaction of *unc-101* with other genes in the vulval induction pathway. Vulval differentiation in approximately twenty animals per each genotype was examined using Nomarski optics. A wild-type animal has three VPCs induced. Fewer than three VPCs induced result in a vulvaless phenotype. More than three VPCs usually result in a normal vulva and a pseudovulval tissue.

[1]data of Aroian and Sternberg, 1991.

[2]data of Han, Aroian and Sternberg, 1990.

Genotype	*unc-101(+)* VPCs/animal forming vulva tiss.	*unc-101(+)* % animals w/>3 VPCs	*unc-101(sy108)* VPCs/animal forming vulval tiss.	*unc-101(sy108)* % animals w/>3 VPCs
+	3	0	3	0
lin-3(e1417)	0.8	0	1.4	0
lin-3(n378)	0.8	0	2.1	0
let-23(sy97)	0	0	0.06	0
let-23(sy12)[1]	0.018	0	2.9	13
let-23(sy1)	0.8	0	3.6	44
let-23(n1045) 15°	1.1	0	0.2	0
let-23(n1045) 20°	2.5	10	0	20
let-23(n1045) 25°	3.4	45	2.0	0
lin-2(n768)	2.8	0	3.2	20
lin-2(e1309)	0.5	0	3.4	35
lin-7(e1413)	0.9	0	3.4	40
lin-10(e1439)	0.5	0	3.4	30
sem-5(n2019)	0.5	0	nearly inviable	
let-60(sy100 dn)[2]	0	0	0	0
let-60(n2021)	2.5	0	inviable	
lin-45(sy96)	0.9	0	1.9	7

Table 2. Complementation of *unc-101* mutations by pJL5 : Suppression of lethality. Eggs were transferred to new plates from hermaphrodites of three different genotypes, and the number of surviving adults and rolling animals were counted after three days . The number of rolling animals represent the stability of the transgene. pJL5 plasmid, when maintained in the transgenic animals, can enable the animals to survive (See text for details).

Genotype of parent	Number of eggs picked	Number of viable adults	viability	Number of rollers	Stability of the transgene
+; Ex[pJL5 +rol-6]	390	354	91%	161	41%
sy108/+; Ex[pJL5 + rol-6]	261	256	98%	79	31%
sy108; Ex[pJL5 + rol-6]	241	173	72%	76	32%
*sy108**	91	43	47%	0	N/A

* Unc animals from the transgenic line, which has lost the transgene.

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Chapter 4

Synthetic interactions of *unc-101* and *sli-1*

Prepared for submission to Science

D-2

Redundant Negative Regulators of the *C. elegans let-23* EGF Receptor Homolog

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In *C. elegans*, induction of the vulva by the somatic gonad is mediated by a signal transduction system that uses nematode homologs of mammalian signal transduction components including *let-23*, an EGF Receptor homolog. We have identified two redundant negative regulators of this process, *unc-101* and *sli-1*. Vulval precursor cells in animals bearing mutations of both the *unc-101* and *sli-1* genes are apparently hypersensitive to the inductive signal leading to a Multivulva phenotype. This Multivulva phenotype requires functional ligand, receptor, and *lin-45(raf)*. *unc-101* and *sli-1* might thus function primarily to negatively regulate the response to the inductive signal.

Vulval induction in *C. elegans* is the result of the production of an inductive signal and the reception and interpretation of this signal by the epidermal vulval precursor cells (VPCs) (1). Several genes absolutely essential for this process have been identified genetically. These include *lin-3*, which encodes an Epidermal Growth Factor (EGF) family member (2), *let-23* (an EGF Receptor homolog) (3), *sem-5* (5) (homologous to GRB2[4]), and *let-60* (ras) (6). Most mutations at these loci confer a vulvaless, or Vul phenotype (Figure 1A). Thus, the signalling process in *C. elegans* vulval induction is very similar to that studied in mammalian systems (7). Several genes, most notably *lin-15*, act to prevent induction in the absence of inductive signal (8,9). Here we describe two genes, *unc-101* and *sli-1*, that function to negatively regulate the responsiveness to the inductive signal. In

a screen for suppressors of a weak mutant allele of *let-23*, the candidate receptor for the inductive signal, we recovered mutations at several loci, including *unc-101* and *sli-1* (10). A mutation at either of these loci confers no vulval induction defect in the absence of a mutation at a second locus, such as the *let-23* locus. *unc-101* mutations have been identified in a number of screens and are pleiotropic. Mutations of *unc-101* confer an inability to move properly, neural defects, male tail defects, and suppression of weak mutations affecting vulval development (11). *sli-1* is a novel silent locus that maps to the left arm of the X chromosome (12) and is defined as a suppressor of cell lineage defect. A mutation at either of these loci is sufficient to suppress the weak *let-23* allele *sy1* (13) from a less than wild-type responsiveness to the inductive signal to a greater than wild-type responsiveness to the inductive signal (Table 1). Doubly mutant animals homozygous for both *let-23* and *sli-1* (14) mutations average nearly four VPCs forming vulval tissue (15), as do double mutants of the genotype *unc-101; let-23* (16). This response is dependent on the presence of inductive signal. If the gonad of these mutant animals is ablated by laser microbeam irradiation in early larvae (before the inductive events occur), no residual vulval differentiation is seen (Table 1). The vulval differentiation seen in *let-23* mutant animals is also dependent on the presence of inductive signal (Table 1). Thus, the products of these two loci act as negative regulators of *let-23*, since a mutation at either locus increases the sensitivity of the *sy1* product, either by increasing responsiveness or increasing the amount of signal present.

Mutations at these loci (*unc-101* or *sli-1*) confer no vulval induction defect on an otherwise wild-type homozygote (i.e., singly mutant animals of either genotype *unc-101* or *sli-1* have wild-type vulvae; Table 1). However,

unc-101; sli-1 double mutant animals (17) often display a greater than wild-type response to the inductive signal (Table 1, Figure 2). Specifically, thirteen of twenty animals displayed greater than wild-type vulval differentiation and the average extent of vulval differentiation was 125% (or an average of slightly less than four VPCs per animal forming vulval tissue; 100% is three VPCs forming vulval tissue). Thus, the negative regulation of *let-23* by these genes is at least partially redundant. In contrast to the animals described above, this vulval differentiation is at least partially gonad independent. If the gonad is ablated in animals of this genotype, there is ~60% residual differentiation (Table 1).

unc-101 and *sli-1* most likely negatively regulate the inductive signalling pathway at the *let-23* step acting on ligand-stimulated activity of the pathway (Figure 1B). The residual differentiation observed in *unc-101; sli-1* double mutants is at least partially dependent on functional *let-23* (EGF receptor). In particular, gonad-ablated triple mutant animals (*unc-101; let-23; sli-1* (18)] display less residual differentiation than do gonad-ablated *unc-101; sli-1* double mutant animals (33% vulval differentiation versus 62% vulval differentiation; Table 1, Figure 2). These suppressor mutations do not simply bypass the requirement for *let-23* (19). This negative regulation is probably acting at the *let-23* step. If this negative regulation acted at a later step in signal transduction (i.e., controlling *let-60* ras), it would be independent of a mutation at *let-23*. This excess differentiation is also at least partially dependent on *lin-3*, the candidate inductive signal. Specifically, animals of the genotype *unc-101; lin-3; sli-1* (20) display a phenotype nearer wild-type than either *lin-3* animals or *unc-101; sli-1* animals. In contrast, a mutation at the *lin-45raf* (21) locus is epistatic to this

phenotype, as triple mutant animals of the genotype *unc-101; lin-45; sli-1* (22) display less than wild-type vulval differentiation (71%, n=27), while the single mutant *lin-45* displays 40% vulval differentiation (23). Thus, this negative regulation occurs (genetically) before the action of *lin-45*, but after or near *let-23* EGF-Receptor and the inductive signal. The *unc-101* and *sli-1* gene products could be acting to control the fates of the VPCs by preventing the spontaneous, signal independent activity of the receptor for the inductive signal (acting similarily to *lin-15* [24]), or as a more specific antagonist of ligand induced signalling, or both. Since the formation of vulval tissue in these multiply mutant animals still requires both functional inductive signal and *let-23*, we conclude that these two gene products primarily function to antagonize the response to the inductive signal, but that they may also play a minor role in lowering the basal signal independent activation of the receptor. If these genes acted only to antagonize this basal activity, the residual differentiation would be independent of the presence of the inductive signal (9).

Based on further analysis of the patterns of vulval differentiation in gonad-ablated animals, we believe that the residual differentiation observed in these gonad-ablated double and triple mutants reflects an earlier use of the inductive signal in another tissue, rather than spontaneous activation of the receptor in the absence of ligand. In gonad-ablated *unc-101; sli-1* double mutants and gonad-ablated *unc-101; let-23; sli-1* triple mutant animals the residual vulval differentiation is strongly shifted to the posterior VPCs (Figure 3). This shift is more noticeable in the *unc-101; let-23; sli-1* triple mutant. We speculate that this residual differentiation is the result of hypersensitivity of the VPCs to inductive signal and leakage of an inductive

signal from an earlier signalling event, perhaps in the early larval tail. Specifically, both *lin-3* and *let-23* are involved in the specification of the P12 neuro-ectoblast fate (25). If the VPCs are hypersensitive, or this signal is overproduced or remains undegraded, they might respond to this perduring signal. The hybrid lineages of the *unc-101; let-23; sli-1* triple mutant animals are consistent with this interpretation. Hybrid lineages are the result of a VPC producing one daughter that displays a vulval fate and one daughter that displays an epidermal fate. Previously, it was shown that animals of the genotype *let-23* often produce VPCs that undergo hybrid lineages. Furthermore, these lineages show a strong tendency to orient such that the daughter forming vulval tissue is nearer the anchor cell (the inducing cell) and the daughter forming epidermal tissue is more distal to the anchor cell than its sibling (26). In the gonad-ablated *unc-101; let-23; sli-1* triple mutants, there are six apparent hybrid lineages; all six are oriented such that the "vulval" daughter is posterior and the "epidermal" daughter is anterior (Figure 3). This is consistent with our interpretation that the residual differentiation in these animals is the result of the inductive signal leaking from a posterior area.

We have described the role of two genes in the regulation of stimulated receptor activity. These two genes, *unc-101* and *sli-1*, are redundant negative regulators of an EGF mediated signalling pathway that is very similar to pathways previously described in mammalian systems (reviewed in 7). In *C. elegans*, several genes (such as *lin-15*) have been described that function to prevent spontaneous activity of the signalling pathway in the absence of the inductive signal. *unc-101* and *sli-1* function to down regulate or desensitize the EGF-receptor (*let-23*) to the inductive signal

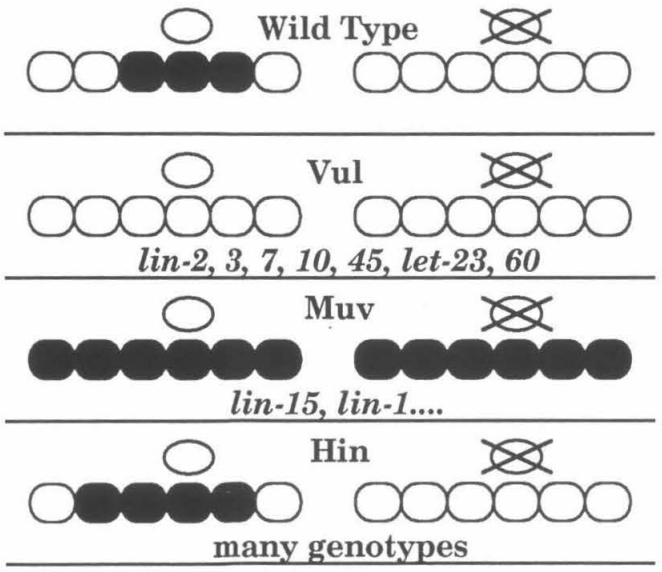
but do not play a major role in regulating the basal activity of the receptor in the absence of ligand. It is possible that either *unc-101* or *sli-1* function in the tissue producing the inductive signal rather than the tissue that responds to the inductive signal, or in a third tissue that functions to destroy signal. In any case, such a negative regulatory function would be important when cells must sense the amount of ligand present, as is likely the case during *C. elegans* vulval induction, or when cells respond to a brief stimulus and then return to a quiescent state after the inductive signal is no longer present in the environment. If higher organisms have redundant negative regulators like *sli-1* and *unc-101*, they might be difficult to identify by molecular genetic methods, since mutation of one gene may not cause an obvious transformation of fate (28).

Figure 1

A. Schematic of representative vulval fates in various classes of mutant genotypes. Vulvaless (Vul) mutations reduce the number of VPCs which respond to the inductive signal and are dependent on the signal. Multivulva (Muv) mutations increase the number of VPCs which respond to the inductive signal and are independent on the signal. Hyperinduction is defined as an increase in the number of VPCs that respond to the inductive signal and are dependent on the signal.

B. Two possible interpretations of the epistatic interactions of the genes discussed. Other interpretations cannot be ruled out without stronger *lin-45* alleles.

Figure 1.A.


Wild Type
Vul
lin-2, 3, 7, 10, 45, let-23, 60
Muv
lin-15, lin-1....
Hin
many genotypes

B.

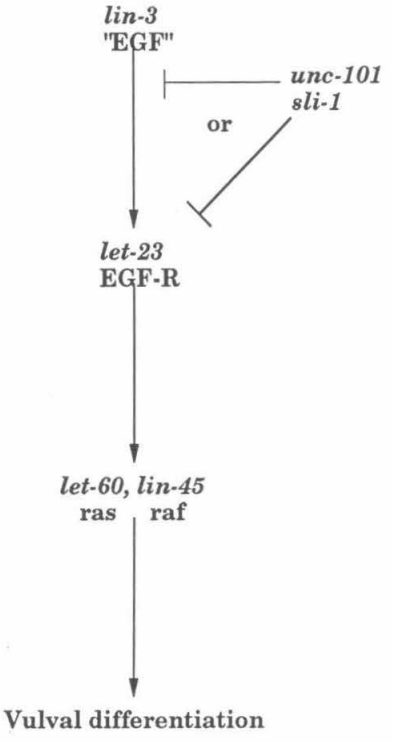

lin-3
"EGF"
unc-101
sli-1
or
let-23
EGF-R
let-60, lin-45
ras raf
Vulval differentiation

Figure 2

Nomarski photomicrographs of representative L3 molt hermaphrodites of genotypes *unc-101(+); sli-1(+). unc-101(-); sli-1(+), unc-101(+); sli-1(-)* and *unc-101(-); sli-1(-)*. Cells assuming vulval fates are grouped by presumptive lineage history. Cells which normally would undergo epidermal fates but are undergoing vulval differentiation are marked in white.

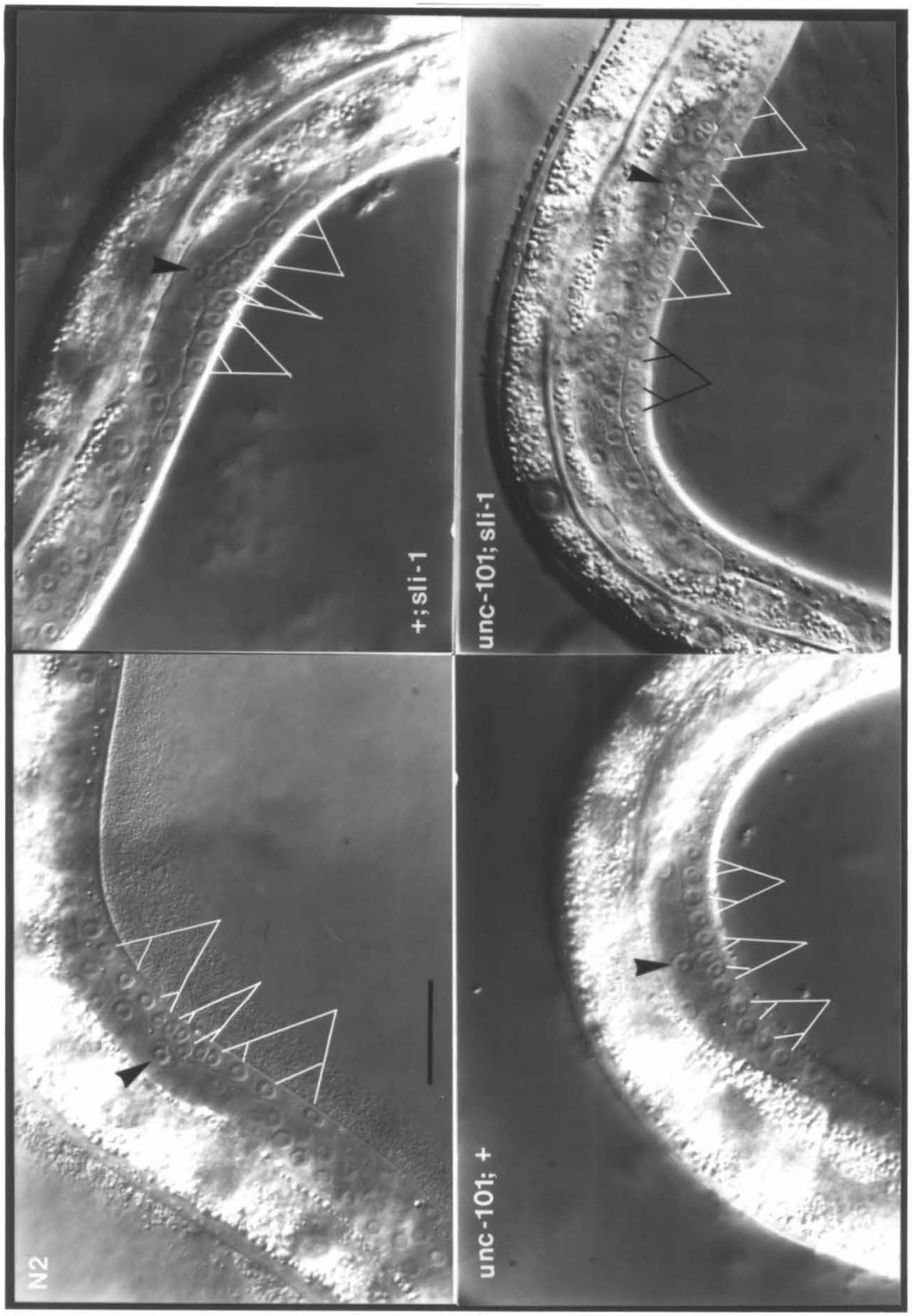

N2
+; sli-1
unc-101; +
unc-101; sli-1

Figure 3

Bar graphs displaying the cumulative patterns of differentiation in several animals of various genotypes. All animals are homozygous for *unc-101* and *sli-1*. The *y* axis is fraction of cells at a given position (i.e., P4.p) forming vulval tissue expressed as a percentage of cells observed at that position.

Figure 3

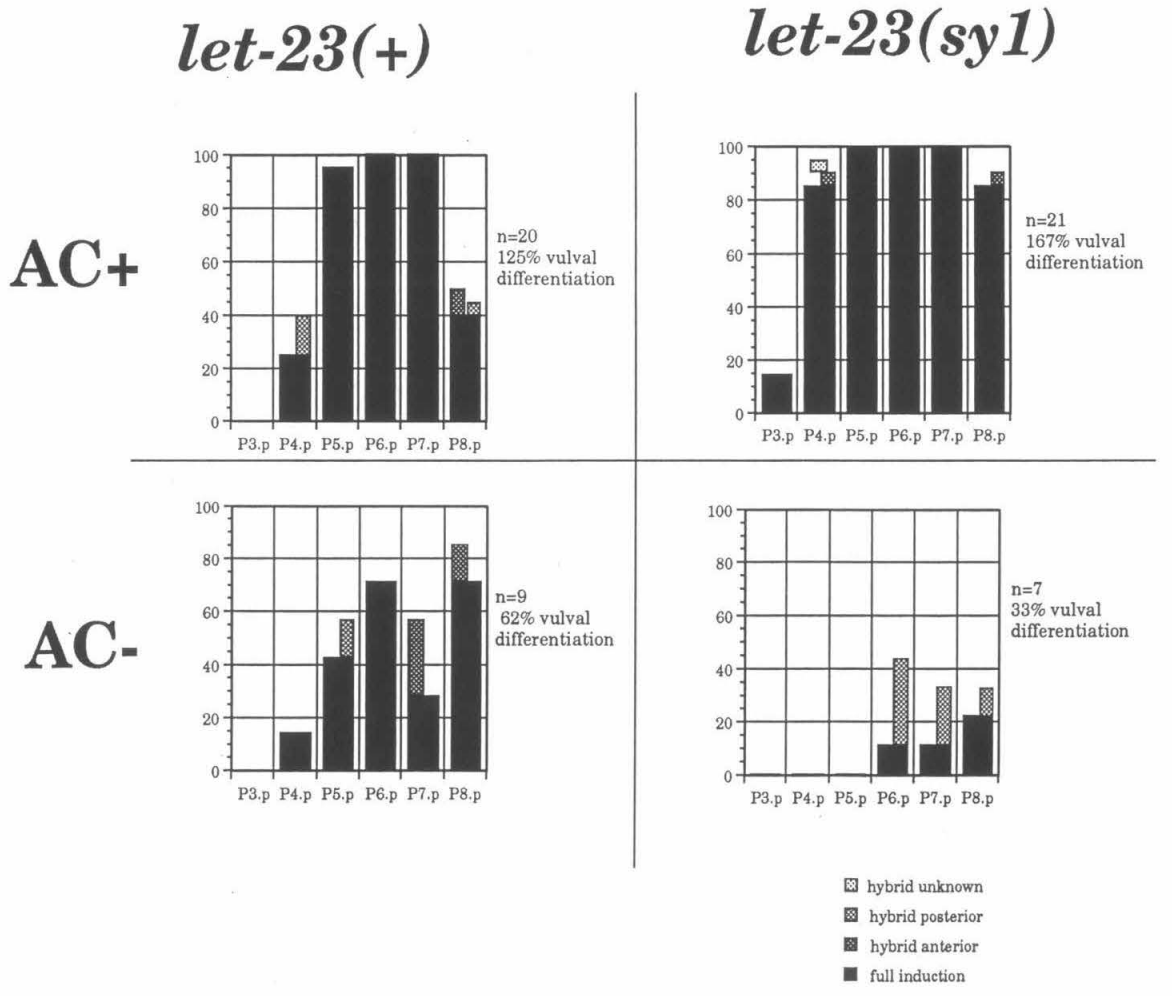

let-23(+)
let-23(sy1)
AC+
AC-
n=20
125% vulval
differentiation
n=21
167% vulval
differentiation
n=9
62% vulval
differentiation
n=7
33% vulval
differentiation
P3.p P4.p P5.p P6.p P7.p P8.p
0
20
40
60
80
100
hybrid unknown
hybrid posterior
hybrid anterior
full induction

Table 1

Extent of vulval differentiation in single, double, and triple mutant animals. Vulval differentiation is measured using Nomarski differential interference contrast microscopy in L3 molt or young L4 animals (6, 10). 100% vulval differentiation is wild type, and this represents three of the six VPCs forming vulval tissue while the remaining three VPCs assume hypodermal fates. Gonad ablations were performed by ablating the cells Z1, Z2, Z3, and Z4 in the early L1 larva (27).

Genotype				Extent of Vulval Differentiation (%)	
lin-3 "EGF"	*let-23* EGF-R	*unc-101*	*sli-1*	gonad$^+$	gonad$^-$
+	+	+	+	100 *(many)*	0 *(many)*
−	+	+	+	26 *(22)*	ND
+	−	+	+	28 *(20)*	0 *(7)*
+	+	−	+	100 *(20)*	0 *(6)*
+	+	+	−	100 *(20)*	0 *(6)*
−	+	−	+	78 *(24)*	ND
−	+	+	−	67 *(20)*	ND
+	−	−	+	118 *(25)*	0 *(7)*
+	−	+	−	144 *(20)*	0 *(7)*
+	+	−	−	125 *(20)*	62 *(9)*
−	+	−	−	96 *(20)*	ND
+	−	−	−	167 *(20)*	33 *(7)*

Table 1.

1. H. R. Horvitz, P. W. Sternberg, *Nature* 351, 535-541 (1991).

2. R. J. Hill, P. W. Sternberg, *Nature* 358, 470-476 (1992).

3. R. V. Aroian, M. Koga, J. E. Mendel, Y. Ohshima, P. W. Sternberg, *Nature* 348, 693-699 (1990).

4. E. J. Lowenstein, *et al.*, *Cell* 70, 431-442 (1992).

5. S. G. Clark, M. J. Stern, H. R. Horvitz, *Nature* **356**, 340-344 (1992).

6. M. Han, P. W. Sternberg, *Cell* 63, 921-931 (1990).

7. J. Schlessinger, A. Ullrich, *Neuron* 9, 383-391 (1992).

8. E. Ferguson, H. R. Horvitz, *Genetics* 123, 109-121 (1989).

9. E. L. Ferguson, P. W. Sternberg, H. R. Horvitz, *Nature* **326**, 259-267 (1987).

10. This screen, the suppressors recovered, and the genetics of these loci will be described elsewhere.

11. J. Golden and D. Riddle, pers. comm.; E. Hedgecock and D. Hall, pers comm.; H. Chamberlin, pers. comm.; S. Emmons, pers. comm.; J. Lee, G. Jongeward, and P. Sternberg, in prep.

12. G. Jongeward and P. Sternberg, in prep.

13. This allele of *let-23* is defective in vulval induction but is not defective in other aspects of *let-23* function. R. V. Aroian, P. W. Sternberg, *Genetics* 128, 251-267 (1991).

14. The *let-23(sy1); sli-1(sy143)* strain was constructed as follows: *let-23(sy1); him-5(e1490)* males were mated to *sli-1(sy143)* hermaphrodites. Cross progeny males were picked and mated to L4 (virgin) *sli-1(sy143)* hermaphrodites. L4 hermaphrodite progeny (apparent cross-progeny) of this mating were selected and allowed to self fertilize. Animals bearing the *let-23(sy1)* mutation segregated animals displaying vulval abnormalities. These

animals were used to establish *let-23(sy1); sli-1(sy143)* stocks.

15. Extent of vulval induction is scored as in M. Han, R. Aroian, P. W. Sternberg, *Genetics* 126, 899-913 (1990).

16. The *unc-101(sy108) let-23(sy1)* strain was constructed by standard methods.

17. The *unc-101(sy108); sli-1(sy143)* strain was constructed by mating heterozygous *unc-101(sy108)*/+ males to *sli-1(sy143)* hermaphrodites. Cross progeny males were mated to L4 (virgin) hermaphrodites. L4 hermaphrodite progeny (apparent cross) of this mating were selected and allowed to self fertilize. Animals homozygous for the *unc-101(sy108)* mutation were selected and used to establish the double mutant strain.

18. The *unc-101(sy108); let-23(sy1); sli-1(sy143)* triple mutant strain was constructed by mating *let-23(sy1); him-5(e1490); sli-1(sy143)* males to *unc-101(sy108); sli-1(sy143)* hermaphrodites. Non Uncoordinated (cross progeny) L4 hermaphrodites were picked and allowed to self-fertilize. *let-23* was homozygosed in the next generation by selecting animals which displayed vulval abnormalities but were not uncoordinated. These animals were allowed to self-fertilize and *unc-101* was homozygosed by selecting uncoordinated animals.

19. Neither an *unc-101* nor a *sli-1* mutation is sufficient to suppress a *let-23* null allele (Jongeward and Sternberg, in prep., J. Lee, G. Jongeward, and P. Sternberg, in prep.).

20. The *unc-101(sy108); lin-3(n378); sli-1(sy143)* strain was constructed in the same manner as the *let-23* triple mutant in note #18. The constituent doubles were also constructed as described for *let-23* (see notes #14 and 16).

21. M. Han, A. Golden, and P. Sternberg, unpub. obs.

22. The *lin-45* strains were constructed in the as the *let-23* strains (see notes 14, 16, and 18), except that *lin-45(sy96) unc-24(e138)*/++ males were used instead of homozygous males.

23. M. Han and P. Sternberg unpub. obs. and our own observations.

24. Strong*lin-15* mutations render vulval development independent of the inductive signal. Gonad-ablation in a *lin-15* animal fails to suppress the Muv phenotype (see # 9). Therefore we conclude that *lin-15* acts to prevent activity of *let-23* in the absence of signal. *lin-15* imay also function as a negative regulator of stimulated activity as well.

25. R. V. Aroian, P. W. Sternberg, *Genetics* 128, 251-267 (1991), R. Hill and P. Sternberg, unpub. obs.

26. R. V. Aroian, P. W. Sternberg, *Genetics* 128, 251-267 (1991).

27. P. W. Sternberg, *Nature* 335, 551-554 (1988).

28. Some nematode strains for this study were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. Supported by USPHS grant HD23690 toP.W.S. P. W. S. is an investigator of the HHMI.

Chapter 5

Characterization of the role of *lin-2, lin-7* and *let-23* in the negative regulation of vulval differentiation

E-2

Genes required for both positive and negative regulation of *C. elegans* vulval induction

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ABSTRACT

***C. elegans* hermaphrodites homozygous for certain alleles of *lin-2*, *lin-7*, and *let-23* display greater than wild-type responsiveness to the inductive signal during vulval induction (the Hyperinduced or "Hin" phenotype). All three gene products are required for response to the inductive signal; in their absence, animals are vulvaless. Because these Hin alleles are recessive and act as if they have a level of activity less than a wild-type allele but greater than a null allele, we believe that these are reduction-of-function alleles. We propose that all three gene products are involved in both positive and negative regulation of vulval differentiation, since reduction-of-function alleles in each can cause an increase, as well as a decrease, in response to the inductive signal. Animals homozygous for two Hin alleles of two different genes are vulvaless, suggesting that the individual Hin alleles encode products that retain relatively more ability to positively regulate than to negatively regulate the response to the inductive signal.**

INTRODUCTION

Vulval induction in the *Caenorhabditis elegans* hermaphrodite involves the production of an inductive signal by a cell in one tissue, the anchor cell of the somatic gonad, and a developmental response to this signal by three of the six Vulval Precursor Cells [VPCs, P(3-8).p] (Fig. 1a and 1b) (Kimble 1981; Sternberg and Horvitz 1986), which are in a different tissue, the

hypodermis. These three VPCs divide and their progeny cells eventually form the vulva, whereas the other three VPCs fuse with the large hypodermal syncytium, hyp7 (Sulston and Horvitz 1977). Mutations which perturb this highly reproducible pattern formation process have been isolated and studied (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Greenwald *et al.* 1983; Ferguson and Horvitz 1985; Ferguson *et al.* 1987; Sternberg and Horvitz 1989; Kim and Horvitz 1990; Beitel *et al.* 1990; Han *et al.* 1990; Aroian and Sternberg 1991). These mutations generally cause one of two phenotypes. Genes such as *let-23, let-60, lin-2, lin-3, lin-7* and *lin-10* are required for the VPCs to assume vulval fates and can be mutated to cause a Vulvaless (Vul) phenotype, in which most or all of the VPCs fuse with the hypodermal syncytium and no vulva is formed (Fig. 1c). Other genes, such as *lin-1* and *lin-15*, are required to prevent VPCs from adopting vulval fates, perhaps by promoting the fusion of the VPCs and the hypodermal syncytium (e.g., Herman and Hedgecock 1990). Loss-of-function mutations in these genes cause a Multivulva (Muv) phenotype in which most or all of the six VPCs, instead of the usual three, assume the vulval fate (Fig. 1d) independent of the inductive signal (Fig. 1e). Generally, animals homozygous for mutations in these genes have functional vulvae as well as ectopic vulval tissue, the latter visible as ventral "pseudovulvae" (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985). A third phenotype, that of Hyperinduction (Hin, Fig. 1f), has been described in animals bearing certain *let-23* alleles (Aroian and Sternberg 1991). Hyperinduction is defined as

more than the wild-type number of VPCs responding to the inductive signal, in other words, a signal-dependent Muv phenotype (Fig. 1h).

Phenotypes similar to Hyperinduction can result when receptors such as the *S. cerevisiae* α-factor receptor and the *E. coli* aspartate receptor are mutated in domains that are involved in negative regulation (Konopka *et al.* 1988; Reneke *et al.* 1988; Russo and Koshland 1983). *let-23* encodes a tyrosine kinase of the EGF-receptor subfamily and is proposed to be the receptor for the inductive signal (Aroian *et al.* 1990). Based on genetic analysis of *let-23*, Aroian and Sternberg (1991) argued that Hin mutations of *let-23* are reduction-of-function mutations, and therefore that *let-23* controls both positive and negative regulation of the inductive process.

Here we describe the genetic analysis of Hin mutations of *lin-2* and *lin-7* (Ferguson and Horvitz 1985). We wished to determine whether Hin alleles of *lin-2* and *lin-7* are genetically similar to the putative null alleles of these genes (Ferguson and Horvitz 1985) or are genetically opposite (i.e., gain-of-function alleles if the putative null alleles are true nulls; for example ectopic expressors or overexpressors of the wild-type or an altered gene product). It was important to investigate these questions, since if these alleles are genetic opposites, then *lin-2* and *lin-7* are likely to be involved in signal transduction but not necessarily involved in negative regulation. On the other hand, if these Hin mutations are genetically similar to the putative null alleles, then *lin-2* and *lin-7* are involved in both signal transduction and negative regulation (e.g., lateral inhibition among VPCs, desensitization of the receptor in the responding cell, or down regulation of receptor or signal).

The functional relationships of *lin-2* and *lin-7* with respect to *let-23* are unknown. An understanding of the relationships among *lin-2* and *lin-7* and the cloned loci *let-60*, a *C. elegans ras* homolog (Han and Sternberg 1990), *let-23*, a *C. elegans* EGF-receptor homolog (Aroian *et al.* 1990), and *lin-10* (Kim and Horvitz 1990) may elucidate how the genes *lin-2*, *lin-7,* and *lin-10* interact with *let-60* and *let-23*.

We demonstrate that these *lin-2* and *lin-7* alleles are genetically similar to the putative null alleles because 1) they are recessive, 2) they act as if they have a level of activity less than a wild-type allele but greater than a putative null allele and 3) animals homozygous for both an amber-suppressible putative *lin-7* null allele and an amber suppressor can be Hyperinduced. These Hin alleles act as if they are weak alleles of the same type as the putative null alleles, rather than a genetic opposite. Therefore, we argue that *lin-2*, *lin-7,* and *let-23* control both positive and negative regulation of the inductive process either independently or as a property of the pathway in which they act. Because the apparent null phenotype of these genes is the absence of vulval differentiation (in the absence of any gene function), we propose that the negative regulation modulates the positive regulation. Animals homozygous for two Hin alleles display a more penetrant Vul phenotype, which is consistent with the hypothesis that these alleles are similar to the puative null alleles.

MATERIALS AND METHODS

General: Standard culture media and conditions were used (Brenner 1974; Wood 1988). The genetic markers used in this study were as follows:

LGII: *rol-6(e187)* (Brenner 1974), *let-23(n1045)* (Ferguson and Horvitz 1985) *let-23(sy1)* (Aroian and Sternberg 1991) *unc-4(e120)* (Brenner 1974), *lin-7(n308, n701, n760,* and *e1413)* (Ferguson and Horvitz 1985), *unc-52(e444)* (Brenner 1974); LGIII: *dpy-18(e364)* (Waterston 1981), LGV: *him-5(e1467* and *e1490)* (Hodgkin *et al.* 1979); LGX: *lin-2(n105, n167, n768, e1453,* and *e1309)* (Ferguson and Horvitz 1985), and *sup-7(st5)* (Waterston 1981).

All strain construction was performed at 20°, except for strains which carried *sup-7(st5)*, which were constructed at 23°.

Strain construction: Strains were constructed according to standard methods (Brenner 1974).

lin-7; lin-2 double mutants were constructed as follows: Males of the genotype *lin-7(x); him-5(e1490)* [where *x* = *n308* or *e1413*] were mated to hermaphrodites of the genotype *unc-52(e444); lin-2(y)* [where *y* = *n768* or *e1309*]. NonUnc hermaphrodite cross progeny were picked individually and single Egl (egg-laying defective, presumably Vul) nonUnc hermaphrodites were picked from their progeny and placed onto individual plates. NonUnc animals were picked from broods which segregated *unc-52* animals but no males [presumptive maternal genotype: *lin-7(x)* +/+ *unc-52(e444); +/him-5(e1490) or +; lin-2(y)*]. Animals which segregated neither Uncs (in their progeny) nor males (in their grandprogeny) were assumed to be of the genotype *lin-7(x); lin-2(y)*.

let-23; lin-2 doubles were constructed as follows: *let-23(sy1* or *n1045); him-5(e1490* or *e1467)* males were mated to nonEgl *rol-6(e187); lin-2(y)* hermaphrodites (where *y* = *n768* or *e1309*). nonRol hermaphrodite cross

progeny were picked and placed individually on plates where they were allowed to self-fertilize. From their progeny, Egl (presumed Vul) nonRol animals were picked and allowed to self-fertilize. From broods which included Rols but no males (presumptive genotype + *let-23*/*rol-6* +; +/*him-5?; lin-2*/*lin-2* where the exact genotype for *him-5* is unknown) Egl animals were picked, allowed to self-fertilize, and animals which segregated neither Rols in their progeny nor males in their grandprogeny were saved (presumptive genotype *let-23; lin-2*).

let-23 lin-7 doubles were constructed as follows: N2 males were mated to *let-23(n1045* or *sy1) unc-52(e444)* hermaphrodites. Male cross progeny were mated to *unc-4(e120) lin-7(z)* hermaphrodites (where *z* = *n308* or *e1413*). NonUnc cross progeny [putative genotype + *unc-4(e120) lin-7(z)* +/*let-23(n1045* or *sy1)* + + *unc-52(e444)* or ++++] were plated individually. From broods in which Egl Unc-52 animals were present, Egl nonUnc-4 nonUnc-52 animals were picked individually. These animals were either *let-23* + *lin-7*/*let-23 unc-52* + or *let-23* + *lin-7*/+ *unc-4 lin-7*. Several Egl nonUnc animals were picked individually from the progeny of these animals. True breeding Egl nonUncs were inferred to be of the genotype *let-23 lin-7*.

Heterozygous *hin*/*"null"*, +/*"null"*, and +/*hin* animals were constructed by the following crosses [where the Hin alleles are *let-23(n1045), lin-7(n308),* and *lin-2(n768)*and the "null" alleles are *let-23(sy1), lin-7(e1413)* and *lin-2(e1309)*]:

let-23(n1045)/+: N2 males were mated to *let-23(n1045) unc-4(e120)* hermaphrodites and nonUnc hermaphrodites were examined.

let-23(sy1)/+: *let-23(sy1); him-5(e1490)* males were mated to *unc-4(e120)* hermaphrodites and nonUnc hermaphrodites were examined.

let-23(sy1)/let-23(n1045): *let-23(sy1); him-5(e1490)* males were mated to *let-23(n1045) unc-4(e120)* hermaphrodites and nonUnc hermaphrodites were examined.

lin-2(n768)/+: N2 males were mated to *rol-6(e187); lin-2(n768)* hermaphrodites and nonRol hermaphrodites were examined.

lin-2(e1309)/+: N2 males were mated to *rol-6(e187); lin-2(e1309)* hermaphrodites and nonRol hermaphrodites were examined.

lin-2(n768)/lin-2(e1309): N2 males were mated to *lin-2(e1309)* hermaphrodites. Cross progeny males were mated to *rol-6(e187); lin-2(n768)* hermaphrodites and nonRol hermaphrodites were examined.

lin-7(e1413)/+: males of the genotype *lin-7(e1413); him-5(e1490)* were mated to *unc-4(e120)* hermaphrodites and nonUnc hermaphrodites were examined.

lin-7(n308)/+: N2 males were mated to *unc-4(e120) lin-7(n308)* hermaphrodites and nonUnc hermaphrodites were examined.

lin-7(n308)/lin-7(e1413): males of the genotype *lin-7(e1413); him-5(e1490)* were mated to *unc-4(e120) lin-7(n308)* hermaphrodites and nonUnc hermaphrodites were examined.

lin-7(e1413) unc-52(e444); sup-7 was constructed by mating *lin-7(e1413) unc-52(e444)/+ +* males to *dpy-18(e364); sup-7unc-6(e78)* hermaphrodites. Cross progeny that segregated Unc-52 were picked. From the progeny of these animals, Unc-6 nonUnc-52 animals which segregated Unc-52 were picked [presumptive genotype: *lin-7(e1413) unc-52(e444)/++; dpy-18?; sup-*

7 unc-6(e78)]. The genotype of *dpy-18* is impossible to determine because *sup-7* is a dominant suppressor of *dpy-18*. Unc-52 animals were picked from the progeny of these animals [presumptive genotype *lin-7(e1413) unc-52(e444); dpy-18?; sup-7 unc-6(e78)*]. The homozygosity of *sup-7* was confirmed by placing 10-15 L3 siblings at 15°. If all were nearly sterile, *sup-7* was assumed to be homozygous since *sup-7* is a cold-sensitive sterile. The homozygosity of *lin-7(e1413)* was confirmed by measuring differentiation of animals grown at 23°, since *sup-7* does not suppress well at high temperatures.

Extent of vulval differentiation: Vulval differentiation was measured as described in Han *et al.* 1990 using Nomarski differential interference contrast microscopy. L3 molt and young L4 hermaphrodites were picked from plates of animals of the relevant genotype and placed in S basal on an agar pad (Wood 1988). Syncytial nuclei in the ventral cord were counted and fates of the VPCs were inferred based on the positions of these nuclei and on the position of vulval nuclei (Sulston and Horvitz 1977; Sternberg and Horvitz 1986; Han *et al.* 1990). Wild-type differentiation was considered to be either 11 or 12 syncytial nuclei, depending on whether P3.p divided. Differentiation is measured as the number of VPCs dividing more than once (thus displaying a non-hypodermal fate), divided by three, which is the number of VPCs which divide more than once in the wild type. In Tables 1 - 3, differentiation is reported as the average differentiation of n (number observed) animals.

Cell ablation: Using a laser microbeam system (Avery and Horvitz 1987; Sternberg 1988), either the cells Z1 and Z4 or Z1, Z2, Z3, and Z4 (the

anchor cell is from either the Z1 or Z4 lineage, Z2 and Z3 give rise to the germline) were ablated in the L1 larva (Sulston and White 1980).

RESULTS

The hyperinduced phenotype: Ferguson and Horvitz (1985) noted that a significant percentage of animals homozygous for certain alleles of the Vul genes *lin-2, lin-7,* and *let-23* [most strikingly *let-23(n1045)* at 25°, *lin-7(n308)* at 25°, and *lin-2(n768)* at 20° and 25°] are Muv, having ventral pseudovulvae in addition to functional vulvae (Fig. 1f, Fig. 2). The putative null phenotypes of *lin-2* and *lin-7* are Vul (Ferguson and Horvitz 1985). *let-23* is also required for induction since animals bearing hypomorphic alleles of *let-23* in *trans* to a deficiency of the locus are Vul (Aroian and Sternberg 1991). Aroian and Sternberg (1991) have shown that for *let-23(n1045),* the extent of vulval differentiation is greater than 100%. They observed that this differentiation is signal-dependent: if the anchor cell precursors are laser ablated, no VPCs are induced (Fig. 1g, Table 1). This phenotype is referred to as Hyperinduced (Hin) and is distinct from the Muv phenotype, in which vulval development is signal-independent (Ferguson *et al.* 1987). We have found that at certain temperatures, animals homozygous for either *lin-2(n768)* or *lin-7(n308)* can be Hin (Table 1). The differentiation seen in these animals is also signal-dependent: in 13 animals homozygous for *lin-2(n768)* and in 13 animals homozygous for *lin-7(n308)* no differentiation was observed following ablation of the gonad, and hence the anchor cell (Table 1). By contrast, in the presence of an intact gonad, 16 of 40 *lin-2(n768)* hermaphrodites were Hin and 14 of 59 *lin-7(n308)* hermaphrodites

were Hin. These data imply that these animals show the Hin phenotype, rather than a weak Muv (signal-independent) phenotype.

Hyperinduced alleles act like reduction-of-function mutations: The Hin alleles cause a phenotype opposite to that of the putative null alleles. One might expect that these Hin alleles are unlike the putative null alleles since the phenotypes are so different. Hin [*lin-2(n768)* and *lin-7(n308)*] and putative null [*lin-2(e1309)* and *lin-7(e1413)*] alleles of both *lin-2* and *lin-7* are recessive to the wild-type (Tables 2 and 3), as are the Hin and Vul alleles of *let-23* (*n1045* and *sy1* respectively) (Aroian and Sternberg 1991; Table 2c). Animals of the genotype *hin/"null"* are less induced than animals of the genotypes *hin/hin,* +/*hin,* or +/*"null"* but are more induced than animals of the genotype *"null"/"null"* (see below and Table 2).

For *lin-2*, the phenotypes of animals bearing one Hin allele, *n768,* and one "null" allele, *e1309*, show less differentiation than wild-type (Table 2a). The heterozygotes rank as follows (ranked from most to least induced): *lin-2(n768)/lin-2(n768)* > *lin-2(n768)/+* = *lin-2(e1309)/+* = *lin-2(+)/lin-2(+)* > *lin-2(n768)/lin-2(e1309)* > *lin-2(e1309)/lin-2(e1309).* In particular, with wild-type differentiation being 100%, *n768/e1309* animals display 62% differentiation, intermediate between the differentiation of *n768/n768* (110%) and the differentiation of *e1309/e1309* (18%).

Similarly, for *lin-7, n308/e1413* heterozygotes (where *n308* is a Hin allele and *e1413* is a "null" allele) are less induced than wild-type animals. The phenotypes of the various *lin-7* heterozygotes rank as follows (ranked from most to least induced): *lin-7(n308)/lin-7(n308)* > *lin-7(n308)/+* =

lin-7(e1413)/+ = *+/+* > *lin-7(n308)/lin-7(e1413)* > *lin-7(e1413)/lin-7(e1413)* (Table 2b). In particular, *n308/e1413* heterozygotes display 46% differentiation, intermediate to the 107% differentiation of *n308* and the 32% differentiation of *e1413*.

let-23 is also similar (Aroian and Sternberg 1991; Table 2c), in that animals of the genotype *let-23(n1045)/let-23(sy1)* are less induced than wild-type, and the heterozygotes rank as follows (ranked from most to least induced): *let-23(n1045)/let-23(n1045)* > *let-23(n1045)/+* = *let-23(sy1)/+* = *+/+* > *let-23(n1045)/let-23(sy1)* > *let-23(sy1)/let-23(sy1)*, where *n1045* is a Hin and *sy1* is a Vul. However, the fraction *n1045/sy1* of animals displaying the Hin phenotype is indistinguishable from the fraction of *n1045/n1045* animals that display the Hin phenotype. Aroian and Sternberg (1991) also found that animals of the genotype *let-23(n1045)/Deficiency* or the genotype *let-23(n1045)/let-23(sy1)* display very little differentiation.

Thus, for *lin-2, lin-7* and *let-23*, Hin alleles are recessive and animals homozygous for a Hin allele are more induced than are animals bearing one Hin allele and one putative null allele. In turn, animals with two putative null alleles are less induced than are heterozygous (*hin/"null"*) animals. Therefore, the Hin alleles act like hypomorphs or recessive neomorphs, and do not act like hypermorphs.

Animals homozygous for a putative amber null allele and an amber suppressor can be Hyperinduced: The putative *lin-7* null allele *e1413* is amber suppressible (Horvitz and Sulston 1980; Ferguson and Horvitz 1985). *sup-7* is a cold-sensitive amber suppressor (Bolten *et al.*

1984) such that animals are most strongly suppressed at 15°, and less strongly or not at all suppressed at 23° (Waterston 1981). Animals of genotype *lin-7(e1413) unc-52(e444); sup-7 unc-6(e78)* were grown at 20°, an intermediate temperature for this suppressor (see below), and examined for their extent of vulval differentiation. Of thirteen *lin-7(e1413) unc-52(e444); sup-7 unc-6(e78)* animals examined, two (15%) were Hyperinduced, ten were wild-type, and one was Vul, while twenty of twenty *lin-7(e1413) unc-52(e444)* animals were Vul. Our observations are consistent with those of Horvitz and Sulston (1980) and Ferguson and Horvitz (1985), since suppression at this temperature (Waterston 1981) should be weaker than the suppression seen at lower temperatures in these earlier studies. This observation is consistent with Hyperinduction being a loss-of-function phenotype, since these suppressed animals should have a level of *lin-7* activity which is greater than that seen in *lin-7(e1413); +* animals, but less than that seen in wild-type animals.

Interactions among Hin and Vul mutations: To further test our hypothesis that the Hin alleles are hypomorphic, we constructed Hin-Hin double mutant animals [the three double mutants *let-23(n1045); lin-2(n768)*, *let-23(n1045) lin-7(n308)*, or *lin-7(n308); lin-2(n768)*]. If these alleles are hypermorphic, then Hin-Hin animals should be hyperinduced or display a novel phenotype, whereas if these animals are not hyperinduced, then the Hin alleles are not hypermorphic.

Animals homozygous for two Hin alleles are less induced than are wild-type animals (Table 3). Specifically, at 23°, with wild-type having 100% vulval differentiation, *let-23(n1045)* has 104%, *lin-2(n768)* has 110%, and

lin-7(n308) has 107%, while both *let-23(n1045); lin-2(n768)* and *let-23(n1045) lin-7(n308)* have no differentiation (0%) and *lin-7(n308); lin-2(n768)* has 55% differentiation. In addition, none of twenty *lin-7(n308); lin-2(n768)* animals were Hin, whereas 40% of animals homozygous for *lin-2(n768)* and 24% of animals homozygous for *lin-7(n308)* were Hin (Figure 3). The decrease seen in the vulval differentiation of the double mutant is a generalized reduction in the amount of differentiation per animal (Figure 3). The *let-23(n1045)* data are less informative regarding the nature of Hyperinduction, since all of the combinations which involve *let-23(n1045)* display highly penetrant Vul phenotypes, with little or no differentiation when examined under Nomarski optics, or on the plate using a dissecting microscope and scoring the Egl phenotype (Table 4). This highly penetrant phenotype is inconsistent with the less severe phenotypes of the double mutant combinations that involve *let-23(sy1)*. These two alleles [*let-23(sy1)* and *let-23(n1045)*] are probably not directly comparable and we therefore do not have a proper control. However, the extent of vulval differentiation of *lin-7(n308); lin-2(n768)* is less than that of control animals [wild type, *lin-7(n308)* and *lin-2(n768)*]. It is likely that the defect in the ability to negatively regulate is masked by the combined defects (of the two alleles) in the ability to positively regulate vulval differentiation in animals of the genotype *lin-7(n308); lin-2(n768)*. Control animals show that mutations in *lin-2* and *lin-7* are partially additive, in that animals homozygous for "null" alleles in these two genes have a more penetrant vulva defect than animals homozygous for either single allele. The extent of vulval differentiation for animals of these genotypes is supported by the dissecting microscope

phenotype of these animals (Table 4). For example, the allele *lin-2(n167)* seems to be a very weak Hin (approximately 4% of the animals resemble Hin animals, although this has not been tested by Nomarski anatomical observations). However, almost all *lin-7(n308); lin-2(n167)* double mutant animals are Vul, and no apparent Hin animals (of 104) were found. This holds true for all of the alleles examined. Specifically, any Hin-Hin double mutant is Vul, rather than wild-type or Hin. These data support the hypothesis that the Hin phenotype is probably caused by a reduction in the ability to properly regulate vulval differentiation, i.e., Hin animals are defective in both positive and negative regulation and that Hin-Hin animals are Vul because the combined defects (of the two alleles) in positive regulation decreases the response to the signal to such an extent that the defect in negative regulation is masked.

A significant fraction (6%) of animals of the genotype *lin-7(e1413); lin-2(n768)* are apparently Hin (nonEgl with ≥ 1 pseudovulva)(Table 4). This is less than the 19% apparent Hin seen for animals of the genotype *lin-2(n768)* and greater than the 2% observed for animals of the genotype *lin-7(e1413)* (Table 4). The extent of vulval differentiation of these doubly mutant animals is consistent with this observation (Table 3) in that these animals are slightly more induced than are animals homozygous for *lin-7(e1413)* but less induced than animals homozygous for *lin-2(n768)*. The simplest interpretation is that this suppression is an allele-specific interaction, which suggests an interaction between these two gene products.

DISCUSSION

We have analyzed the nature of alleles of the Vul genes *lin-2, lin-7,* and *let-23* at the level of individual VPC fates by dosage analysis, gene interaction, and cell ablation. Vul genes such as *lin-2, lin-7*, or *let-23* are clearly required for response to the inductive signal. If alleles of a Vul gene can also confer a Muv or Hin phenotype, then an understanding of the nature of these alleles may allow us to elucidate the role of the wild-type gene product in the inductive process. If the allele is hypermorphic, then the gene is probably only required for the stimulation of vulval differentiation. On the other hand, if the allele is hypomorphic, then the gene is required for both stimulation of vulval differentiation and negative regulation of vulval differentiation, since these alleles reduce the ability of a gene product to function. Based on their genetic behavior, we believe that the Hin alleles of *lin-2* and *lin-7*, like the Hin alleles of *let-23*, are reduction-of-function alleles. Therefore, we propose that *lin-2*, *lin-7* and *let-23* act in the control of both positive and negative regulation of vulval induction, either in series or in parallel. The negative regulation function of *let-23* modulates the positive regulation rather than vice versa, since the null phenotype in the vulva of *let-23* is Vul, showing that an inability to positively regulate can mask a defect in negative regulation (Aroian and Sternberg 1991). This is also true for *lin-2* and *lin-7*, since animals homozygous for putative null alleles are Vul as are animals doubly homozygous for Hin alleles of both *lin-2* and *lin-7*.

A phenotype similar to Hyperinduction has been seen in other receptors which transduce signals and are subject to negative regulation (Konopka *et al.* 1988; Reneke *et al.* 1988; Russo and Koshland 1983). For instance,

certain truncations of the *S. cerevisiae* α-factor receptor are actually hypersensitive to α-factor. To our knowledge, such a phenotype has not been described for genes that interact with these receptors and are required for the signalling process. However, during *C. elegans* vulval development, the products of the genes *lin-2* and *lin-7* are likely to be involved in both of these processes, since animals bearing homozygous mutant alleles of either gene can display a Hyperinduced phenotype and these alleles reduce the activity of their respective genes. It is formally possible that these alleles are recessive neomorphs. However, the fact that an amber-suppressible genetic null allele of *lin-7* can be partially suppressed to give a Hyperinduced phenotype is strongly suggestive that Hyperinduction is indeed a reduction-of-function phenotype.

Perhaps Hyperinduction is a property of the response pathway or a part of that pathway. We propose that reception of inductive signal by VPCs stimulates a signal transduction pathway involving several steps. *let-23*, a candidate receptor, acts in concert with *lin-2, 7, 10* (Figure 4) to activate *let-60 ras*, and also to activate a negative regulatory loop. This loop might act on adjacent cells (e.g., by carrying out lateral inhibition, Sternberg 1988) or on the signal transduction process itself (e.g., down regulation or desensitization). It is likely that the role of *lin-10* is similar to that of *lin-2* and *lin-7* since these genes are indistinguishable by epistasis tests (Ferguson *et al.* 1987; D. Parry, S. Kim, and R. Horvitz, personal communication) and a Hin allele of *lin-10* has been recovered (S. Kim and R. Horvitz, personal communication).

Hypomorphic alleles of genes acting in the positive regulatory loop only (i.e., acting after the pathway branches) should result in a weak Vul phenotype, not a Hin phenotype. Muv alleles of these genes should be hypermorphs, since a gain-of-function should be the only way to increase the number of cells responding to the signal. A good candidate for a gene acting only in the stimulatory pathway is *let-60*, since the Muv alleles of *let-60* are hypermorphic (Beitel *et al.* 1990; Han *et al.* 1990) and *let-60* acts after *lin-2* and *lin-7* by epistasis tests (Han *et al.* 1990; D. Parry, S. Kim, and R. Horvitz, personal communication).

In summary, we believe that *lin-2*, *lin-7* and *let-23* are required for both positive and negative regulation of vulval induction because animals homozygous for certain alleles of these three genes are more induced than wild-type animals. These alleles are apparently hypomorphic, thus implying that they are defective in the ability to modulate response to the inductive signal via negative regulation.

Acknowledgements: We thank Chip Ferguson for his suggestion that *lin-2* and *lin-7* may have Hyperinduced alleles, Bob Horvitz for kindly providing the *lin-2* and *lin-7* alleles used in this study, Stuart Kim, Diane Parry, and Bob Horvitz for sharing unpublished data, Raffi Aroian, Helen Chamberlin, Andy Golden, Min Han, and Russell Hill for helpful discussions, and Paul Herman, Stuart Kim, Howard Lipshitz, Man Lun Yip and members of our laboratory for critical readings of this manuscript. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). P.W.S. is an investigator of the Howard Hughes Medical Institute. This work was supported by grant HD23690 from the U.S. Public Health Service to P.W.S.

FIGURE 1 Vulval induction.

A. Wild-type vulval induction. The anchor cell of the somatic gonad produces an inductive signal which causes three of six VPCs to form vulval tissue (solid black). The other three cells form non-specialized hypodermal tissue (white).

B. If the anchor cell is ablated (x), all six cells form non-specialized hypodermal tissue.

C. Differentiation in a completely Vul animal. All of the VPCs form hypodermal tissue, either due to a defect in signalling or response.

D. Differentiation in a completely Muv animal. All of the VPCs form vulval tissue.

E. Differentiation in a gonad-ablated Muv mutant animal. Differentiation in Muv animals is gonad-independent in that all six VPCs in animals with no anchor cell still assume induced fates.

F. Hyperinduction (representative). In a subset of animals homozygous for Hin alleles, more than three VPCs respond to the inductive signal.

G. Differentiation in a gonad-ablated Hin mutant animal. If the germ-line precursors are ablated in Hin animals, the VPCs all assume the hypodermal fate.

Figure 1.

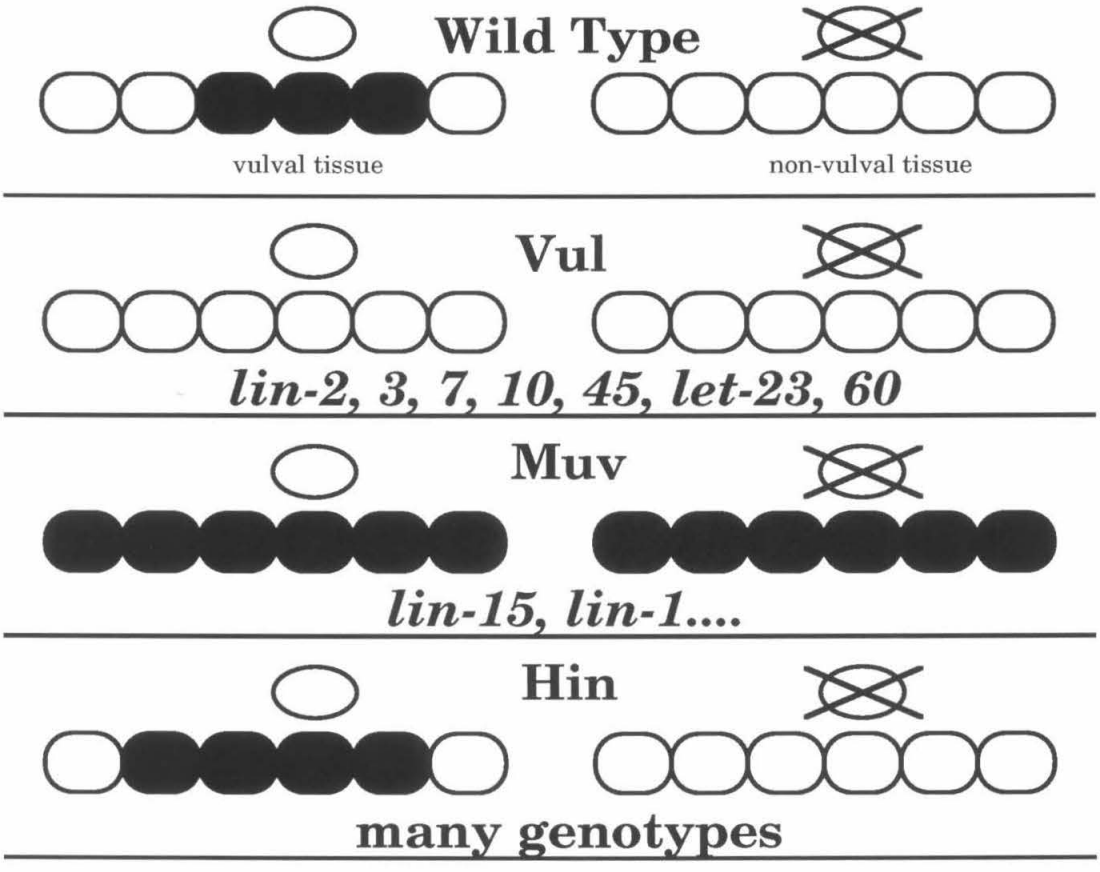
Wild Type
vulval tissue
non-vulval tissue
Vul
lin-2, 3, 7, 10, 45, let-23, 60
Muv
lin-15, lin-1....
Hin
many genotypes

FIGURE 2 Nomarski photomicrographs of a representative *lin-2(n768)* Hin animal. The fates of VPCs in this animal were inferred from anatomical observations; P4.p, P5.p, and P6.p form a wild-type vulva, while both P7.p and P8.p apparently underwent hybrid [S TT] lineages, each of which is scored as 1/2 cell differentiation. Therefore, this animal displays 133% differentiation. **a.** L4 stage. Large white arrow points to the presumptive vulva, small white arrows to the presumptive pseudovulvae, black arrow to P7.pp, a syncytial nucleus, scale bar = 20μm. **b.** Adult. Large arrow points to the vulva, small arrows to the pseudovulvae, scale bar = 20μm.

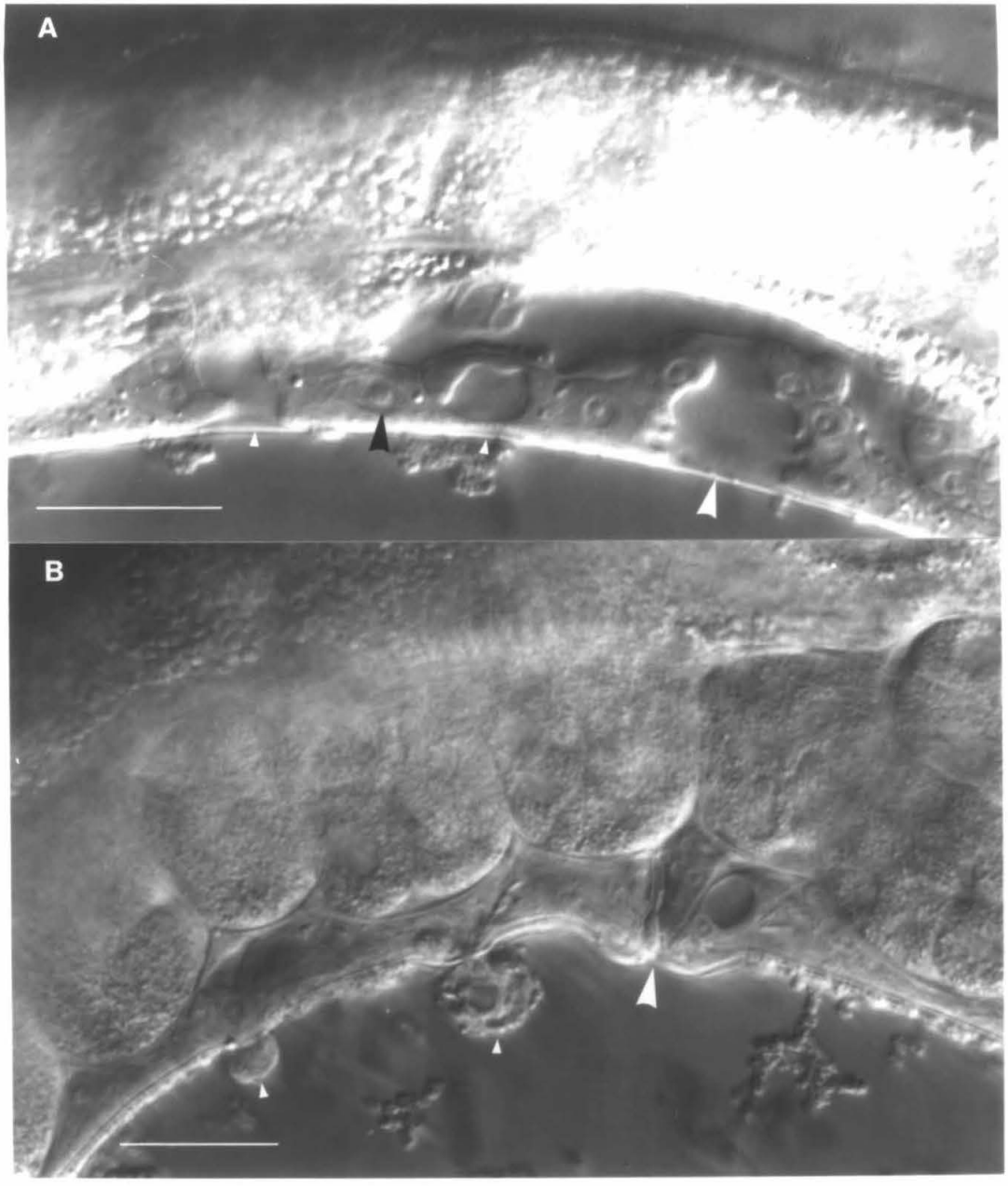
A
B

FIGURE 3 Bar graphs showing the number of cells induced in animals that were examined under Nomarski optics. 3.0 cells induced is wild-type (100% differentiation), individual Hyperinduced animals have > 3.0 cells induced. Striped bars are not to scale.

N2
(wild type)
n=many
23°
100%
let-23(n1045)
n=19
23°
104%
lin-7(n308)
n=59
23°
107%
lin-2(n768)
n=40
23°
110%
lin-7(n308); lin-2(n768)
n=20
23°
55%
let-23(n1045) lin-2(n768)
n=20
23°
0%
let-23(n1045) lin-7(n308)
n=20
23°
0%

FIGURE 4 Models for the roles of *lin-2* and *lin-7* in the vulval induction pathway, incorporating *lin-10, let-23,* and *let-60. let-23* is proposed to act as the receptor for the inductive signal and activate both positive regulation (via *let-60 ras*) and a negative regulatory loop. No order of the action of *lin-2, lin-7,* and *lin-10* is inferred relative to *let-23*. While the Muv phenotype of a strong reduction-of-function allele of *lin-15* is epistatic to the Vul phenotype of *lin-2, lin-7,* or *lin-10* (Ferguson, Sternberg and Horvitz 1987), a less severe allele, *lin-15(n765)* is not epistatic to a *lin-2* mutation (P. Sternberg and A. Holboke, unpublished observations). We believe that the interaction of *lin-2, lin-7, lin-10,* and *let-23* with the Muv genes *lin-15, lin-8,* and *lin-9* is one of degree and quite allele dependent and that these Vul genes cannot be ordered by interaction with these Muv genes. The sites of action of the Vul genes is not known. However, the simplest interpretation is that they act in the VPCs.

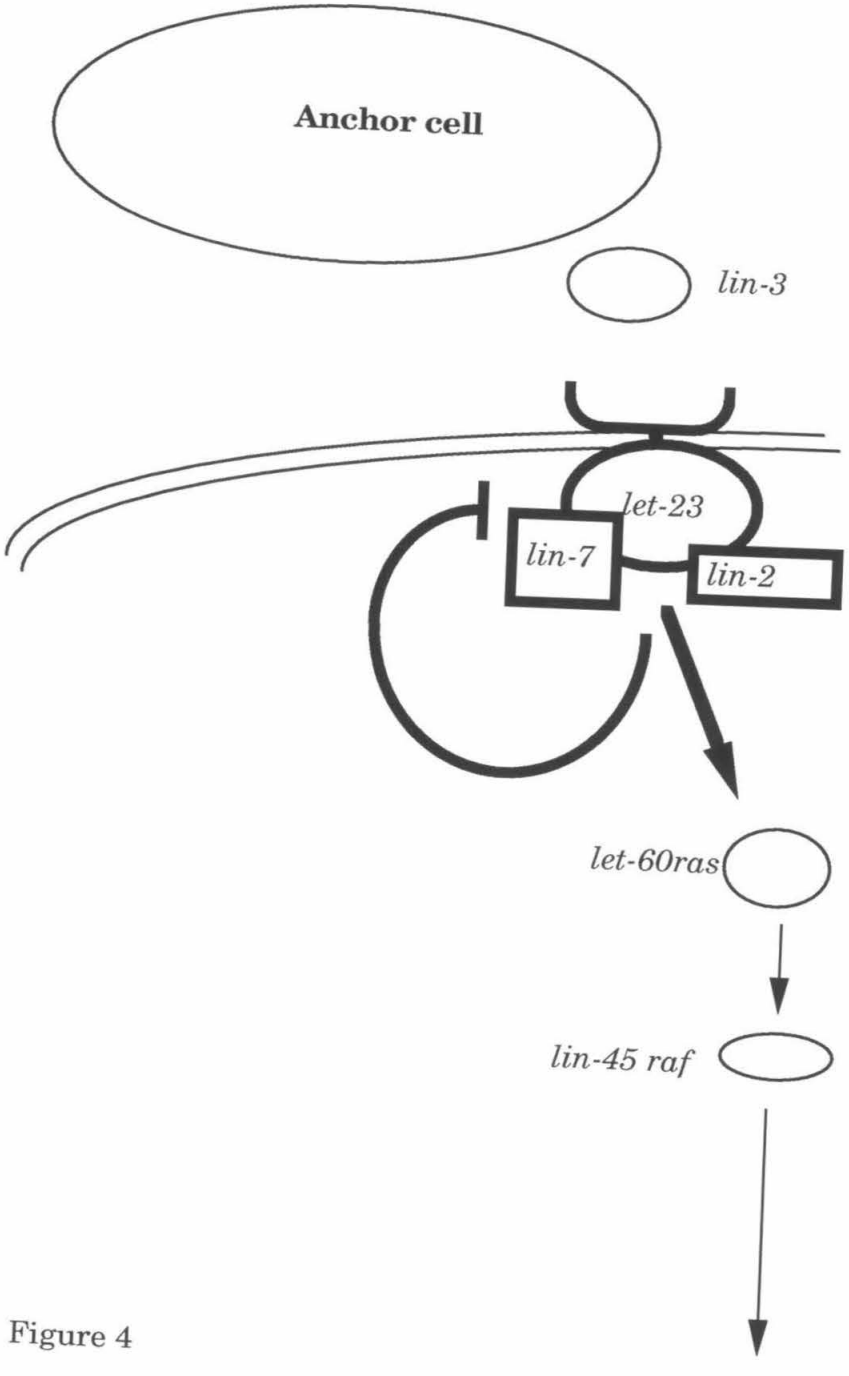
Anchor cell
lin-3
let-23
lin-7
lin-2
let-60ras
lin-45 raf
Vulval fates

Figure 4

TABLE 1 The extent of vulval differentiation of homozygous Hin animals with and without anchor cells. Two of the alleles used in this study, *let-23(n1045)* and *lin-7(n308),* are cold-sensitive (Ferguson and Horvitz 1985). At low temperatures (15°), most animals homozygous for either allele are Vul, while at higher (23°) temperatures, many homozygous animals are Hin. The gonad precursor cells Z1, Z2, Z3, and Z4 or Z1 and Z4 were ablated in L1 larvae homozygous for Hin alleles. When these animals reached the L3 lethargus or L4 stage, the extent of vulval differentiation was scored by anatomy as described in Materials and Methods.

TABLE 1
Extent of vulval induction of Hin homozygotes at various temperatures

Genotype	20°, gonad+		23°, gonad+			23°, gonad-	
	% differentiation	n	% differentiation	% Hin[b]	n	% differentiation[a]	n
+	100	20	100	0	20	0	many
let-23(n1045)	83	20	104	15	20	0	7[c]
lin-2(n768)	96	38	110	40	40	0	13
lin-7(n308)	88	20	107	24	59	0	13

[a] Measured as described in Materials and Methods.

[b] Percent of animals observed which were Hyperinduced

[c] Data of Aroian and Sternberg (1991), ablations were performed at 25°.

TABLE 2 Complementation analysis of Vul and Hin alleles. n, number of animals; [a], measured as described in Materials and Methods.

A. *lin-2* differentiation at 23°. See text for crosses.

B. *lin-7* differentiation at 23°. See text for crosses.

C. *let-23* differentiation at 23°. See text for crosses.

TABLE 2
Extent of vulval differentiation
A. *lin-2* differentiation at 23°

Genotype	% differentiation [a]	% of animals Hin	n
+/+	100	0	20
+/*n768*	100	0	20
+/*e1309*	100	0	20
n768/*n768*	110	40	40
n768/*e1309*	62	5	20
e1309/*e1309*	18	0	20

B. *lin-7* differentiation at 23°

Genotype	% differentiation [a]	% of animals Hin	n
+/+	100	0	20
+/*n308*	100	0	20
+/*e1413*	100	0	20
n308/*n308*	107	24	59
n308/*e1413*	46	0	19
e1413/*e1413*	32	0	20

C. *let-23* differentiation at 23°

Genotype	% differentiation [a]	% of animals Hin	n
+/+	100	0	20
+/*n1045*	100	0	20
+/*sy1*	100	0	20
n1045/*n1045*	104	15	20
n1045/*sy1*	82	10	20
sy1/*sy1*	28	0	20

[a] Measured as described in Materials and Methods.

TABLE 3 The extent of vulval differentiation of animals mutant at two loci involved in vulval development at 23°. n, number of animals; [a], measured as described in Materials and Methods.

A. Extent of vulval differentiation of animals of the genotype *let-23; lin-2*.

B. Extent of vulval differentiation of animals of the genotype *lin-7; lin-2*.

C. Extent of vulval differentiation of animals of the genotype *let-23; lin-7*.

TABLE 3

Differentiation of doubly mutant animals at 23°

A.

			lin-2
let-23	+/+ (n) [a]	*n768/n768* (n) [a]	*e1309/e1309* (n) [a]
+/+	100 (20)	110 (40)	18 (20)
n1045/n1045	104 (20)	0 (20)	0 (20)
sy1/sy1	28 (20)	18 (20)	8 (20)

B.

			lin-2
lin-7	+/+ (n) [a]	*n768/n768* (n) [a]	*e1309/e1309* (n) [a]
+/+	100 (20)	110 (40)	18 (20)
n308/n308	107 (59)	55 (20)	10(20)
e1413/e1413	32 (20)	65 (20)	13 (20)

C.

			let-23
lin-7	+/+ (n) [a]	*n1045/n1045* (n) [a]	*sy1/sy1* (n) [a]
+/+	100 (20)	104 (20)	28 (20)
n308/n308	107 (59)	0 (20)	0 (20)
e1413/e1413	32 (20)	0 (20)	1 (20)

[a]Measured as described in Materials and Methods

TABLE 4 Gross phenotypes of various combinations of mutations at *lin-2, lin-7,* and *let-23*. Phenotypes were observed under low magnification dissecting microscopes.

TABLE 4
Dissecting microscope phenotypes of *let-23*, *lin-2* and *lin-7* mutant animals

Genotype	% Egl	%nonEgl	% nonEgl with ≥ 1 pseudovulva	n
lin-2				
n768	17	83	19	135
n105	46	54	2	52
n167	51	49	4	231
e1453	76	24	0	149
e1309	92	8	0	188
lin-7				
n308	49	51	13	201
n701	56	44	2	194
e1413	82	28	2	173
n760	79	21	1	143
let-23				
n1045 [a]	46	54	7	57
sy1	90	10	0	106
let-23; lin-2				
n1045; n768 [a]	99	1	0	94
n1045; n105 [a]	100	0	0	79
n1045; n167 [a]	100	0	0	104
n1045; e1453 [a]	100	0	0	68
n1045; e1309 [a]	100	0	0	85
sy1; n768	92	8	2	195
sy1; n105	99	1	0	156
sy1, n167	99.5	0.5	0	211
sy1; e1309	94	6	0	141

lin-7; lin-2				
n308; n768	81	19	2	175
n701; n768	89	11	1	208
e1413; n768	78	22	6	98
n760; n768	85	15	2	239
n308; n105	95	5	2	121
e1413; n105	80	20	2	203
n760; n105	88	12	3	174
n308; n167	99	1	0	104
e1413; n167	99.5	0.5	0	218
n760; n167	96	4	1	215
n308; e1453	86	14	4	195
n701; e1453	92	8	2	156
e1413; e1453	97	3	0	278
n760; e1453	91	9	1	150
n308; e1309	98	2	1	178
n701; e1309	98	2	1	200
e1413; e1309	94	6	2	200
n760; e1309	93	7	0.5	217
let-23 lin-7				
n1045 n308 [a]	100	0	0	95
n1045 n701 [a]	100	0	0	52
n1045 e1413 [a]	100	0	0	95
n1045 n760 [a]	100	0	0	54
sy1 n308	99.5	0.5	0	215
sy1 n701	96	4	0.5	200
sy1 e1413	98	2	0.5	197
sy1 n760	93	7	0.7	150

[a] percentage of viable, fertile animals not including dead or sterile animals

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Chapter 6

Discussion

Discussion

We have identified two novel negative regulators of *C. elegans* vulval differentiation (*unc-101* and *sli-1*) and characterized the role that these genes and three other genes (*lin-2, lin-7* and *let-23*) play in the control of negative regulation of this signaling pathway. Animals defective in this negative regulation display excessive vulval differentiation. Animals bearing loss-of-function mutations at any one of these five loci do not display an obvious failure in negative regulation. Rather, mutations at *unc-101* or *sli-1* confer no vulval defect, while mutations at *lin-2* or *lin-7* cause a failure in differentiation of vulval tissue (a failure in positive regulation) and mutations at *let-23* confer an early larval lethal phenotype. The roles of each of these genes in negative regulation is apparent only in mutant backgrounds (*unc-101* and *sli-1*) or in animals bearing hypomorphic alleles (*lin-2, lin-7* and *let-23*). Animals bearing mutations at two of these loci often display a mutant phenotype. Generally this phenotype is consistent with a failure in negative regulation, in that extra cells undergo vulval fates. In some cases, this extra differentiation is dependent on the presence of inductive signal, while in other backgrounds, some cells undergo vulval differentiation despite the ablation of the anchor cell. In any case, most of these genes seem to require the presence of functional *let-23* to act, suggesting that they control the activity of *let-23*, rather than acting as downstream antagonists of *let-23*. Despite the similarity of these genes, it is apparent that they work in very different ways, based on other phenotypes associated with mutations at these loci and molecular identities of the putative proteins encoded by the loci. *unc-101* encodes a clathrin adaptor protein that is presumably involved in regulating the sorting of proteins from the *trans*-Golgi. One would assume

that such a protein would be expressed globally and function in all cells. In contrast, *sli-1* encodes a protein with some similarity to the *c-cbl* proto-oncogene. *c-cbl* is a nuclear protein with a Zinc-finger motif (Langdon et al., 1989), and presumably acts as a transcription factor. *let-23* is a homolog of the EGF-receptor(Aroian et al., 1990). *lin-2* and *lin-7* are uncloned, but are genetically similar to *lin-10*, which is broadly expressed but has no sequence similarity to any known genes (Kim and Horvitz, 1990). The involvement of all of these genes in negative regulation of vulval differentiation suggests that a number of negative regulatory loops impinge on this pathway to refine and limit the number of cells responding to the inductive signal. Of the genes characterized in this study, only *let-23* might have been predicted to act as a negative regulator on the basis of studies on mammalian cells in culture. Biochemical and molecular genetic experiments have identified several regions of the EGF receptor that are required for negative regulation of the response to EGF.

Targets of negative regulation

Genetic evidence suggests that *lin-2, lin-7* and *sli-1* act at or near *let-23* or *sem-5*. The evidence regarding the *unc-101* is inconclusive. A number of pathways of negative regulation act on the EGF-receptor in tissue culture experiments. One might expect that control of this pathway would primarily be at the level of the receptor and its sensitivity to the presence of ligand. Internalization, degradation, and several sites of phosphorylation all act to control the function of the mammalian EGF-receptor. All of these pathways

act redundantly on the receptor to decrease the sensitivity and responsiveness of the receptor to EGF, as well as decreasing the amount of receptor present on the surface of the cell. Such responses must be under tight control, as in a developing animal, it is important to regulate the response to a growth factor. Down regulation of response prevents unnecessary and dangerous proliferation and allows future response to the growth factor under different circumstances. The presence of multiple pathways of negative regulation allows very fine control, as certain stimuli (for instance activation of other receptors) can stimulate a subset of the pathways of down-regulation, allowing the partial desensitization of the receptor without totally preventing further response. *let-23* itself controls at least one negative regulator, as evidenced by the hyper-responsiveness seen in certain allelic combinations. The receptor probably controls many more negative regulators based on experiments with the mammalian receptor. Nearly all of the pathways of negative regulation which act in mammalian cells are activated by the receptor upon stimulation. The same pathways are probably present in *C. elegans* and activated by *let-23*.

The site of action of the negative regulation by *unc-101* is unclear. Some mutations at *let-23* are suppressed, while others are enhanced. Suppression of *let-23* by *unc-101* in other tissues is also variable. Lethality is apparently not suppressed. It is possible that an *unc-101* mutation might suppress the lethality of a weak *let-23* allele. None of the *let-23* alleles which lessen viability are suppressed in any tissue by an *unc-101* mutation. If *unc-101* suppressed one of these alleles in some tissues but failed to suppress the lethality this interaction would be more compelling. As it stands, our assumption is that an *unc-101* mutation is not sufficient to suppress the loss

of *let-23* gene product. Mutations in other genes are suppressed variably as well. *lin-2, lin-7,* and *lin-10* mutations are suppressed. These genes act at or near the *let-23* step. Mutations at *lin-3* and *let-60* are not suppressed, but mutations at *lin-45* are somewhat suppressed. The inconsistency of these interactions makes any placement or model for the action of *unc-101* highly speculative. One hypothesis for this gene is that mutations at *unc-101* result in endosomes that are inefficient at the degradation of the receptor/ligand complex. This might sensitize the cell to the signal, provided that the receptor fails to activate other pathways of desensitization. Interactions with downstream mutations would vary with the quantitative interactions of these mutations . Mutations at other genes which act to control positive and negative regulation would be partially suppressed, while mutations at genes required only or primarily for positive regulation would not be suppressed. Since suppression by *unc-101* seems to generally correlate with very weak mutations, this model may be an accurate prediction of the activity of this gene. One interesting experiment that follows this model is the treatment of cells in tissue culture with Brefeldin A and subsequently assaying the ability of these cells to respond to EGF and the kinetics of down-regulation in these cells. *unc-101* may act in other receptor mediated pathways as well. *unc-101* mutations enhance phenotypes associated with weak *daf-1* mutations (J. Lee, pers. comm.). *daf-1* is a receptor serine/threonine kinase involved in dauer formation. The role of clathrin and its adaptor proteins in signal transduction may be quite general.

sli-1 is a candidate for acting at the *let-23* or *sem-5* step. Mutations at *lin-3, let-60,* and *lin-45* are not suppressed by mutations at *sli-1.* Mutations at *let-23, sem-5, lin-2, lin-7,* and *lin-10* are suppressed. Of these genes, the best

candidate targets are *let-23* and *sem-5*, as *sli-1* mutations suppress *let-23* in all tissues but do not suppress all of the phenotypes associated with mutations at *sem-5*. *lin-2, lin-7,* and *lin-10* display no phenotypes in most of the tissues that *sli-1* acts in. The simplest model is that *sli-1* regulates *let-23* or another gene that acts in all the tissues which require *let-23*. This regulation is dependent on the presence of *let-23*, as only weak alleles of *let-23* are suppressed, suggesting that *sli-1* mutations do not relieve the requirement for *let-23*. This requirement is not changed in animals bearing mutations at both *unc-101* and *sli-1*. These animals display excessive differentiation. This differentiation is dependent on *let-23* as well. Animals bearing mutations at *unc-101, sli-1,* and *let-23* in which the gonad has been ablated display less vulval differentiation than otherwise identical *let-23(+)* animals. *sli-1* may act as a dimer or multimer. One heteroallelic combination (*sli-1(sy102)/sli-1(sy112)*) displays a wild-type phenotype (the lack of suppression) in a *let-23(sy1)* background, suggesting that these two mutations affect different properties of the gene, such that the *trans*-heterozygote is capable of functioning properly. The similarity to an apparent transcription factor suggests that this interaction is not direct, rather that *sli-1* is controlling the transcription of *let-23* or other genes acting in the vulval differentiation pathway. A *sli-1* mutation does relieve the requirement for the interaction of *sem-5* and *let-23*, as mutations which presumably prevent this interaction (*let-23(sy97)* and *sem-5(n2019)*) are suppressed to an apparent wild-type phenotype.

It is possible that *sem-5* is the target of *sli-1* negative regulation in vulval differentiation. Analysis ofthe interaction of *sli-1* with *let-23* gain-of-function mutations and null alleles of *sem-5* might clarify the target of *sli-1* regulation.

If *sli-1* regulates *sem-5* during vulval differentiation, this interaction is tissue-specific. *sli-1 sem-5* double mutant animals are still egg-laying defective, presumably as a result of the sex myoblast migration defects.

lin-2 and *lin-7* may act on the same pathway of negative regulation as *let-23*. Since mutations of these genes all display similar vulval phenotypes, it seems likely that *lin-2* and *lin-7* act to transduce the signal of *let-23* to both positive and negative regulators. It is possible that the targets of these genes are not the same. The simplest explanation is that these genes act on the same targets and act in a linear pathway. Analysis of the epistasis of these genes with dominant mutations or transgenes would bolster this interpretation. Based on the assumption that *lin-2* and *lin-7* activate the same negative regulators as *let-23*, it seems likely that the target of this negative regulation is also *let-23*.

Redundancy of negative regulators

The novel negative regulators described here are redundant. Both *unc-101* and *sli-1* are silent in the vulva until combined with another mutation which is defective in negative regulation. This is similar to what is seen in mutant versions of the EGF-receptor expressed in tissue culture cells. Mutations of single regions or residues important for specific negative regulatory functions generally do not result in the complete failure of negative regulation. Negative regulation is impaired, but not prevented in such cells. Combinations of multiple mutations results in more severe failures in negative regulation. Such an arrangement is sensible for the control of a molecule as potentially hazardous to the organism as a growth factor receptor. Unregulated receptors would result in energetically costly

cell proliferation at best, and cancerous growth in the worst case. Multiple redundant pathways of negative regulation prevent the activation of such a receptor except for short periods of time under carefully controlled conditions, thus preventing overgrowth of the receptor positive cells.

Although a great many of the targets of EGF-receptor are known, only a few are known that act as negative regulators. PLC-γ was identified as an associate of the EGF-receptor; it controls the activation of protein kinase C. This pathway, however, is a relatively minor negative regulator of the receptor compared to internalization. Even the biological significance of the PLC-γ data is unclear. The tyrosine that defines the putative PLC-γ binding site on the receptor has not been shown to be an *in vivo* target of autophosphorylation in a wild-type context. It is certainly possible that the activation of PLC-γ requires only a few receptor molecules per cell to be phosphorylated at this site, and that this level of phosphorylation is undetectable with present techniques. However, PLC-γ is detectable in coprecipitation experiments using anti-EGF-receptor antibodies, suggesting that detectable amounts of this protein are associated with the receptor, and implying that the binding site should be equally phosphorylated. The explanation for this inconsistency is as yet unknown. No genes have been shown to act in the internalization of EGF-receptor except for clathrin. Several genes should act in this process, and these are probably difficult to identify using the biochemical techniques available, especially if these genes do not associate directly with the EGF-receptor.

Genetic screens offer one approach that will identify genes acting in these pathways which might not have been identified by biochemistry and molecular biology. Genetic suppressors and enhancers need not interact

directly to be identified, thus allowing the isolation of mutations in genes that would be difficult to find biochemically. Once these mutations have been recovered, it is a simple matter to examine their interactions with other genes involved in the pathway of interest as well as their interactions with other suppressors and enhancers. Such analysis has allowed us to show that the *unc-101; sli-1* double mutant is hypersensitive to the inductive signal. Such a phenotype should be recoverable as a transforming mutation in mammalian cells, but the frequency of double mutations makes this difficult, and methodologies for recovering the mutation responsible for a transformed phenotype strongly biases toward dominant mutations.

Recent experiments have shown that other genes may act redundantly with *sli-1*. In a screen for new *sli-1* alleles, an unlinked non-complementing locus, defined by a single allele, *sy262*, was identified. Mutations at this locus recessively suppress the starting *let-23* mutation. Animals heterozygous for both *sy262* and *sli-1* (genotype *let-23(sy1)/let-23(sy1); sy262/+; sli-1(sy143)/+*) also display suppression of the *let-23* allele to hyperinduction. Genetically, this mutation is similar to *sli-1*. Mutations at this locus are silent suppressors of *let-23,* and confer only a slight head abnormality on animals bearing the mutation in an otherwise wild-type background. Further analysis of this gene and its role in negative regulation of the vulval differentiation pathway will ascertain whether this gene acts in the same loop as *sli-1* or defines yet another negative regulatory loop.

One question regarding *sli-1* and *unc-101* is whether either of these mutations define the negative regulator that is controlled by *let-23*. A mutation in this pathway should suppress some of the *let-23* mutations. For instance, *let-23(sy1)* should be suppressed, perhaps to give a hyperinduced

phenotype. However, the hyperinduced phenotype of *let-23(n1045)* at 25° should be enhanced or unchanged. No combination of *let-23* mutant allele with such a mutation should give only wild-type animals. Such a phenotype suggests the existence of some form of negative regulation. Neither *unc-101* nor *sli-1* fits these criteria. *unc-101* mutations make *let-23(n1045)* animals more defective in vulval differentiation. This is inconsistent with these two mutations acting in the same pathway. *sli-1* seems even less likely. Ninety-six of ninety-eight animals of genotype *let-23(sy97); sli-1(sy143)* are wild-type in the extent and pattern of vulval differentiation. This is unlike the patterns seen in *let-23(n1045)* animals at 25°, which is the expected pattern for an animal bearing a mutation in the negative regulator controlled by *let-23*. However, a screen for hyperinduced animals in a *let-23(sy97); sli-1(sy143)* genetic background might allow the recovery of mutations in this negative regulator.

A speculative model for the activity of *sli-1*

sli-1 displays several interesting genetic properties. One model for the role of this gene in the regulation of vulval differentiation is based on these behaviors. *sli-1* mutations do not suppress null mutations at *let-23*, a homolog of the EGF receptor. *sli-1* mutations suppress weak mutations of *let-23* in all tissues. Therefore *sli-1* acts as a modifier of *let-23* activity. *sli-1* suppresses *let-23* but does not suppress mutations at *let-60 ras* or *lin-45 raf*, suggesting that *sli-1* acts before these genes. *lin-2, lin-7,* and *lin-10* are suppressed by *sli-1* but mutations in these genes do not affect tissues in which *sli-1* displays suppression ability. The simplest explanation for this is that *sli-1* acts on *let-23* or the pathway which includes *let-23*, rather than *lin-*

2, lin-7, or *lin-10.*

The activity of *sli-1* is predicted by the interaction of *sli-1* mutations with the *let-23* mutation *sy97* and *sem-5*. *let-23(sy97)* is predicted to encode a truncated version of Let-23. The most notable residues lacking in this truncation are the predicted Sem-5 binding sites at the extreme C-terminus. These are the homologs of sites which are relevant in the mammalian EGF receptor and are good matches for the consensus site defined for binding to the SH2 domain present in the *sem-5* gene product(Sonyang et al., 1993). This mutation is suppressed to wild-type (in all tissues) by a strong *sli-1* allele. Most other *let-23; sli-1* double mutant animals undergo excessive vulval differentiation. *let-23* is required for both negative and positive regulation of vulval differentiation. Therefore the *let-23(sy97)* allele is capable of activating this negative regulation, but *sli-1* is not required for the negative regulation activated by *let-23*. A strong (non-null) *sem-5* mutation is also suppressed to very nearly wild-type vulval differentiation by this *sli-1* allele. A subset of the phenotypes associated with this *sem-5* mutation are suppressed. For instance, the sex myoblast migration abnormalities are not suppressed by *sli-1*. This migration does not require *let-23*. Thus, in the vulval induction pathway, *sli-1* renders animals independent of *sem-5* but not *let-23*. One model for the function of *sli-1* is that *sli-1* antagonizes *sem-5* by interacting with components of the signal transduction pathway which are specific to the vulval and not the sex myoblastpathways. For instance, *sli-1* may activate GAP activity. *let-60 ras* does not seem to be required for sex myoblastmigration, suggesting that these cells may utilize another G protein, such as *rap*. If this is true, then the GAP activity required in these cells would also be different. *sli-1* could also act to prevent activation of a GNEF

by *sem-5* in the VPCs and may not act in the sex myoblasts. Alternatively, *sli-1* might act as a repressor of *sem-5* transcription in the vulval precursor cells. Mutations at *sli-1* would derepress this transcription, allowing the production of more *sem-5*. This might compensate for mutations which lower the ability of Let-23 and Sem-5 to interact. This model can be confirmed by examining the phenotypes of animals homozygous for null alleles of *sem-5* and *sli-1* and animals bearing *let-23* gain-of-function alleles and extra copies of *sli-1(+)*.

How many negative regulators?

Clearly there are several independent pathways that negatively regulate Let-23 in vulval development. The pathway defined by *lin-15* et al. functions to prevent *lin-3*-independent receptor activity. *let-23* activates yet another pathway of negative regulation. Presumably, this activation acts via *lin-2* and *lin-7*. Based on similarities to the mammalian receptor, one might expect that this pathway is activated in response to stimulation but does not function to lower the basal activity of the receptor. A third pathway of negative regulation presumably acts through *sli-1*. This negative regulation seems to primarily act as a down-regulator of stimulated receptor, since ablation of the inductive tissue prevents any vulval differentiation. If *sli-1* acted to prevent ligand independent activation of Let-23, then some vulval differentiation should occur in the absence of the inductive signal. A fourth possible pathway is revealed by *unc-101* mutations. This role is tentative, as mutations at *unc-101* enhance one *let-23* allele, suggesting that the interaction of these genes may be less obvious than a simple negative regulation. *sy262* may define yet another negative regulator of *let-23*. It

seems more likely that this gene acts in the same pathway as *sli-1*. This pathway is clearly dose-sensitive, as heterozygous deficiencies of the *sli-1* region will suppress some *let-23* genotypes. Given this, standard non-complementation screens will select for mutations in other genes acting in the same pathway. Additional alleles mapping to the *sy262* locus and the interaction of mutations at this locus with the genes involved in vulval differentiation will determine if this gene acts similarly to *sli-1*

Comparison to the screens in *Drosophila*

Another issue is the sensitivity of the starting mutations to suppression. Are the screens carried out here similar to those used for suppressors and enhancers of *sevenless*? At this point, it is difficult to conclude one way or another. No candidate GAP mutation has been recovered as a *let-23* suppressor. However, only a single mutation recovered as a suppressor of a weak *sevenless* mutation has been described. Other mutations recovered may be *Drosophila* homologs of *unc-101* or *sli-1*. Gain-of-function mutations at *Son of sevenless* were recovered as suppressors of a stronger *sevenless* allele. The only true dominant mutation recovered in these screens was a *let-60 ras gf*. It is possible that similar mutations might be recoverable as suppressors of *let-23*. As yet, molecular searches for the *C. elegans* homolog of this gene have proven fruitless. The differences between the *Drosophila* gene and the *C. elegans* gene may prevent recovery in either search.

Gene number and silent mutations

Early estimates for the number of genes in *C. elegans* were based on classical genetic methods and estimation of the number of genes which could mutate to give a visible phenotype. These estimates, 2,000 (Brenner, 1974),

to 3,500 (Clark et al., 1988), are at odds with the preliminary estimates of 15,000 genes based on genomic sequencing (Sulston et al., 1992) and analysis of cDNAs. Estimates from this approach suggest that the gene number is significantly higher. One of the apparent causes for such a discrepancy is genes such as *sli-1*. Mutations in *sli-1* would not have been recovered as visible mutations in such a screen and subsequently would have been uncounted. This extends to a number of classes of genes for *C. elegans*, including nearly all of the loci involved in eating, chemotaxis, and mechanosensation, as well as silent suppressor and enhancer loci and the genes acting in the *lin-15* pathway of negative regulation. Silent suppressors of *glp-1*, *lin-10, lin-11* and *lin-1* have been identified but have not yet been fully characterized. It is clearly very difficult to estimate gene number based on visible phenotypes, as the definition of visible phenotype is a very elusive item indeed.

Silent suppressors are frequently regarded as uninteresting loci when recovered. Such an approach can lead one to ignore interesting and relevant genes, since many genes which are the focus of a great deal of attention in molecular biology have proven to have very subtle phenotypes when mutated in genetic systems. Redundant gene products are likely candidates for such silent suppressors and redundancy often reflects the functional importance of a pathway, rather than its irrelevance.

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Appendix: Other mutations that I have characterized

In the course of the work described in the text, I have characterized several other mutations. Some of these are of interest, others are simply markers at this point.

sy61. This allele causes a recessive Egl phenotype. The locus maps to the left arm of L.G. 5 and is uncharacterized. I have not determined whether imipramime or that other drug are able to confer egg laying competence on these homozygotes.

sy90. This allele causes semi-dominant suppression of *let-23(sy1)*. Double homozygotes can display the Hin phenotype. This allele is unmapped.

suv-1(sy107, sy113, sy133, and *sy120)*. Some of the mutations recovered as suppressors of *let-23* are apparently alleles of *suv-1*, a suppressor identified by mutations that suppress a strong *lin-10* phenotype from Vul to apparently Hin. These alleles are tentatively assigned to this complementation group on the basis of three-factor crosses. When placed in *trans* to *unc-1(e719) dpy-3(e27)*, these mutations were picked up by Unc nonDpy recombinant chromosomes but not by Dpy nonUnc recombinant chromosomes. *sli-1* is to the left of *unc-1*, *suv-1* is to the right of *unc-1*. Finer mapping would be required to unambiguously identify these mutations as *suv-1*. This is further

complicated by the semi-dominance of some *suv-1* alleles in some genetic backgrounds,

At least some mutations at this locus also interact with *unc-101* Three of twenty-four animals of genotype *unc-101(sy108); suv-1(sy133)* and one of seventeen animals of genotype *unc-101(sy108); suv-1(sy120)* displayed a Hin phenotype.

Examinations of *sli-1(sy143)suv-1(n1329)* doubly mutant animals showed that one of twenty animals observed displayed a Hin phenotype. This *suv-1* allele displayed no synthetic interaction with *unc-101.*

sy260. This mutation was originally recovered (as an Egl- line) by Anna Newman in her screen for somatic gonad abnormalities and has been mapped to the right arm of linkage group I (some mapping was done by Helen Chamberlin). Animals homozygous for this mutation display a highly penetrant Vul phenotype. It is possible that this is maternally rescued to some extent. If a heterozygote is allowed to self-fertilize and apparent Vuls are selected, a fraction of these animals lay a few eggs before bagging. These Egl+/- animals reliably segregate all Egl- self-progeny, suggesting that these animals are indeed homozygous. These animals are probably slightly more induced than homozygous daughters of homozygous mothers. Given the penetrance of the Egl- phenotype, there appears to be a fairly high level of vulval differentiation. I would estimate that about 1/4 to 1/3 of animals bear vulval tissue. There may be other defects associated with this mutation that make the Egl- phenotype so penetrant. This phenotype is also very difficult to suppress. I have carried out three mutageneses for suppressors of the Egl-

phenotype. A very rough estimate of the extent of this screen is 20,000 mutagenized haploid genome sets. No suppressors were recovered in these screens.

This gene acts late in the vulval induction pathway. Animals doubly homozyogus for this allele and a trimethyl psoralen induced allele of *lin-1* are Muv and very unhealthy. This strain proved impossible to maintain. Andy Golden has shown that a *lin-45(+)* transgene on an extra-chromosomal array is not sufficient to rescue this phenotype. Helen Chamberlin has shown that homozygous males are defective in their spicules.

This mutation is similar to other mutations at a locus being characterized by both Min Han's lab and Bob Horvitz's lab. Complementation tests suggest that these mutations define the same locus. These groups have identified mutations conferring similar phenotypes and mapping ot the right arm of L.G. I as suppressors of *let-60 ras gf* alleles.

sy262. This mutation was recovered serendipitously in the *sli-1* non-complementation screen. This screen was detailed in chapter 2. Briefly, *let-23(sy1); him-5(e1490)* males were mutagenized under standard conditions and subsequently mated to *let-23(sy1); che-2(e1033) sli-1(sy143) unc-1(e719)* hermaphrodites. F1 Egg laying competent nonUnc or Muv nonUnc progeny were placed on individual plates and allowed to self-fertilize. From the progeny of these animals, several Muv nonUnc hermaphrodites were selected and allowed to self-fertilize on individual plates. Animals which segregated neither Uncs nor Ches (and were therefore homozyogus for the mutagenized paternal X chromosome) were kept (those segregating either marker were

discarded). Several of the progeny of these *let-23(sy1) / let-23(sy1); him-5(?); sli-1(new) / sli-1(new)* animals were cloned individually to fresh plates to recover a Him line. Such a line was recovered. Hermaphrodites form this line were examined under Nomarski optics and vulval differentiation was observed.

Males of this strain were mated to *unc-1(e719) dpy-3(e27)* hermaphrodites as a backcross. nonDpy nonUnc cross progeny (F1) were selected and allowed to self-fertilize. From the F2 progeny, Hin nonDpy nonUnc animals were selected and allowed to self-fertilize on individual plates. Some of these animals segregated Dpy Uncs. A Hin Dpy Unc was selected to allow further analysis. A Hin Dpy F2 animal had been observed, suggesting that all was not normal. This animal was not selected as it was assumed that this was simply an animal displaying a rare pattern of induction seen in *let-23(sy1)* homozyogotes. These animals occasionally display several pseudovulvae but have no vulva, resulting in and egl- apparent Hin (at the cellular level, these animals probably have three or less VPCs forming vulval tissue). To determine if these loci (*sy262, unc-1* and *dpy-3*) were truly unlinked, *let-23(sy1); him-5(e1490)* males were mated to the *let-23(sy1); sy262; unc-1(e719) dpy-3(e27)* hermaphrodites. nonDpy nonUnc L4 hermaphrodites were selected and allowed to self-fertilize on fresh plates. The self-progeny of these animals were observed. If *sy262* is unlinked to the left arm of X, then Hin nonDpy nonUnc animals will be frequent (about 1/4 of animals). If *sy262* is linked to the left arm of X, then Hin nonDpy non Unc animals will be rare (less than 5% of all animals). Hin nonDpy nonUnc animals were very frequent, suggesting that *sy262* is unlinked to X.

This locus was recovered as a new mutation which failed to complement *sli-1(sy143)*. This observation was rechecked. *let-23(sy1); him-5(e1490) sy262* males were mated to *let-23(sy1); che-2(e1033) sli-1(sy143) unc-1(e719)* hermaphrodites. nonUnc cross progeny were examined and appeared to be suppressed and Hin. Subsequent mapping suggests that the *sy262* mutation is on L.G. 5, possibly within the cluster.

In the course of mapping this mutation, we have learned the following: (1) animals homozyogus for this mutation in a *let-23(+)* background are apparently wild-type. 56/56 Rol progeny of a *rol-6(e187)* +/+ *let-23(sy1); sy262 him-5(e1490)*/+ + hermaphrodite were non Muv. (2) this mutation is semidominant, animals of genotype *let-23(sy1)/let-23(sy1); sy262*/+display a phenotype intermediate to that of animals of genotypes *let-23(sy1)/let-23(sy1); +/+* and *let-23(sy1)/let-23(sy1); sy262/sy262*. Further characterization of the locus will determine if this gene acts in a similar manner to *sli-1*. As mutations at both *sli-1* and *sy262* confer a notchhead phenotype (see Figure 6, chapter 2).

sy257 We had been interested in extending the series of experiments regarding the sensitivity of P3.p to the inductive signal in the absence of any other VPCs. Paul had shown that if P(4-8).p were laser ablated in the late L1/early L2, P3.p would sometimes fail to divide (~50% of animals), or undergo one of the three vulval fates. I attempted to extend this observation. We planned to determine the fraction of isolated P3.p's undergoing specific fates in operated N2 animals as a control for similar isolations in animals bearing mutations resulting in Hin phenotypes. I killed the P(4-8).p cells in

nine animals. In eight animals, P3.p uncerwent vulval differentiation. In the remaining animal, P3.p underwent the tertiary fate. In no instance did P3.p fail to divide. Since about 50% of P3.p's were expected to assume the non-VPC fate, we examined the parental strain. This strain was an isolate of the wild-type strain N2 that had been maintained by matings since September of 1987. In these animals, P3.p always divides to generate two daughters. This strain was backcrossed by mating males bearing this putative mutation (the strain had been maintained as a male hermaphrodite strain by daily matings) to *unc-1(e719)*, an easily scorable Unc that does not affect P3.p (about 50% of P3.ps of *unc-1(e719)* animals fail to divide). nonUnc hermaphrodites were selected and allowed to self-fertilize. L4 hermaphrodite progeny were observed under Nomarski optics. Animals with six syncytial cells anterior to the vulva (P3.p had apparently undergone the tertiary fate) were recovered and allowed to self-fertilize on individual plates. The progeny of these animals were subsequently observed. One of eight lines bred "true," segregating only animals in which P3.p divided.