SIGNAL TRANSDUCTION, REGULATION, AND DEVELOPMENTAL LOGIC OF EGFR SIGNALING IN *C. ELEGANS*

Thesis by

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Abstract

RTKs-mediated signaling systems and the pathways with which they interact (e.g., those initiated by G protein-mediated signaling) involve a highly cooperative network that sense a large number of cellular inputs and then integrate, amplify, and process this information to orchestrate an appropriate set of cellular responses. The responses include virtually all aspects of cell function, from the most fundamental (proliferation, differentiation) to the most specialized (movement, metabolism, chemosensation). The basic tenets of RTK signaling system seem rather well established. Yet, new pathways and even new molecular players continue to be discovered. Although we believe that many of the essential modules of RTK signaling system are rather well understood, we have relatively little knowledge of the extent of interaction among these modules and their overall quantitative importance.

My research has encompassed the study of both positive and negative signaling by RTKs in *C. elegans*. I identified the *C. elegans* SOS-1 gene and showed that it is necessary for multiple RAS-mediated developmental signals. In addition, I demonstrated that there is a SOS-1-independent signaling during RAS-mediated vulval differentiation. By assessing signal outputs from various triple mutants, I have concluded that this SOS-1-independent signaling is not mediated by PTP-2/SHP-2 or the removal of inhibition by GAP-1/RasGAP and it is not under regulation by SLI-1/Cbl. I speculate that there is either another exchange factor for RAS or an as yet unidentified signaling pathway operating during RAS-mediated vulval induction in *C. elegans*.

In an attempt to uncover the molecular mechanisms of negative regulation of EGFR signaling by SLI-1/Cbl, I and two other colleagues co-discovered that RING finger domain of SLI-1 is partially dispensable for activity.

This structure-function analysis shows that there is an ubiquitin protein ligase – independent activity for SLI-1 in regulating EGFR signaling. Further, we identified an inhibitory tyrosine of LET-23/EGFR requiring *sli-1*(+) for its effects: removal of this tyrosine closely mimics loss of *sli-1* but not loss of other negative regulator function.

By comparative analysis of two RTK pathways with similar signaling mechanisms, I have found that *clr-1*, a previously identified negative regulator of *egl-15* mediated FGFR signaling, is also involved in *let-23 EGFR* signaling. The success of this approach promises a similar reciprocal test and could potentially extend to the study of other signaling pathways with similar signaling logic.

Finally, by correlating the developmental expression of *lin-3 EGF* to *let-23 EGFR* signaling activity, I demonstrated the existence of reciprocal EGF signaling in coordinating the morphogenesis of epithelia. This developmental logic of EGF signaling could provide a basis to understand a universal mechanism for organogenesis.

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C. elegans vulval development as a model system to study the cancer biology of EGFR signaling

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Introduction

Model genetic organisms have proven useful in the elucidation of cellular regulatory pathways and their normal physiological roles. EGFR is important during animal development in the control of cell growth, differentiation, metabolism, and migration, among many other biological responses. Here we review studies of EGFR signaling in *C. elegans* and mainly focus on its regulation during vulval development.

C. elegans biology and genetics

To grow *C. elegans*, all one needs is an agar plate seeded with *E. coli* to serve as a growth medium, a wire pick to transfer animals from plate to plate for consistent log phase propagation and setting up of genetic crosses, and an incubator to provide an environment of constant temperature (20°C) [1]. Animals can be maintained indefinitely in a frozen state and re-grown after rapid thawing. The short life cycle (three days per generation) makes experiments relatively rapid. One intriguing feature of *C. elegans* is its simple anatomy. Both sexes (males and hermaphrodites) have an essentially invariant cell lineage, and thus an almost constant cell number; the adult male has only 1031 somatic nuclei; the adult hermaphrodite has only 959 [2] [3]. Forward or reverse genetics can both be used very conveniently in *C. elegans* to clone or characterize genes. There are three facts that make forward genetics practical in *C. elegans*. First, there are well-established genetic tools such as genetic markers and balancers distributed throughout the entire genome. Second, 80% of the genome are contained within cosmid pools and the rest 20% of the genome are covered by the YACs, which allows positional cloning to be efficiently done [4]. Moreover, a clone-based

physical map of the *C. elegans* genome has been properly aligned with the existing genetic map. This map allows genetic mapping to pinpoint a small physical region covered by a reasonable amount of cosmids and/or YACs that can be tested for rescuing the mutant phenotype in transgenic worms.

There are several advances that allow facile characterization of a gene function by reverse genetics in *C. elegans*. By microinjection of DNA into germline cytoplasm, we can efficiently transfer gene and stably maintain it as extrachromosomal arrays from generation to generation, which allows us to repeatedly analyze gene function in a specific genetic background after a single injection [5]. Recently, a technical breakthrough allows *C. elegans* experimenters to transiently knockout a particular gene function as desired. Double-stranded RNA has been shown to robustly and specifically reduce, if not remove, the gene expression in several species, including *C. elegans*, *Drosophila*, *Trypanosoma brucei*, mice, and plants [6] [7] [8] [9] [10]. Stable knockout of a single gene can also be performed by use of the chemical reagents. A desired deletion event is detected in a large pool of worms by means of PCR. The pool is consecutively subdivided into many small ones with PCR performed in each step till a single animal is isolated. Lastly, the essential complete of the *C. elegans* genome sequencing project gives us an opportunity to search for the homologs of genes of interest on a genome wide scale, and possibly to estimate the minimal set of players required to serve the biological functions of a signaling pathway.

The characterization of a signaling pathway does not proceed at the same pace among different systems. Therefore, a new component found in one system will boost the motivation to search for the homolog in other systems since we believe evolution favors the conservation of important functions. Until complete genome sequences are available, the failure to identify a homolog of gene of interest is not unusual. However, if we still fail to do so after the finish of

genome sequencing, we should start to consider an alternative mechanism such that the function can be bypassed or there is redundancy.

Signal transmission by RTKs

All RTKs exhibit a similar molecular architecture and apparently are activated by a common mechanism [11] [12]. A general mechanism for ligand-induced activation of RTKs has been established. Ligand binding to the extracellular domain induces receptor dimerization which in turn leads to activation of the catalytic protein kinase domain [13]. Autophosphorylation of tyrosine residues that are located in noncatalytic regions leads to the generation of docking sites for SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains of signaling molecules [14]. These molecules include the adapter GRB-2, which leads to RAS activation, phospholipase C-γ (PLCγ), the tyrosine phosphatase SHP-2, RAS-GTPase activating protein, the regulatory subunit of phosphatidylinositol-3-OH kinase [14] [15] [16], and the proto-oncoprotein c-Cbl [17]. The association between proteins with SH2/PTB domains and pTyr serves as the initial step in the recruitment of an activated RTK signaling complex. Specific pTyr sites are recognized by distinct SH2 and PTB domains [18] [19] [20] [21] [22]. Interaction of RTKs with different substrates is thought to result in activation of distinct signaling pathways, thus producing distinct cellular responses [11] [23]. Excessive or inappropriate signaling from RTKs has been implied in many cases of malignant transformation [24] [25]. Therefore, proper mechanisms must be employed to strongly control the signaling from RTKs to prevent ligand independent activation and attenuate signaling after activation has occurred.

EGF RTK signaling is used multiple times during the normal development of *C. elegans*, mediating vulval induction, viability, male spicule development, hermaphrodite ovulation, and differentiation of the ventral uterus and posterior ectoderm [26] [27] [28] [29] [30] [31]. Vulval induction was among the first processes discovered to be mediated by EGF RTK signaling. The high resolution of scoring the phenotype of vulval induction makes it a very sensitive assay to identify mutants and to reflect the relative strength of RAS-dependent signaling. All the other developmental events are also RAS-dependent, except for hermaphrodite ovulation, which will be discussed later.

EGFR signaling in vulval induction

The wild-type vulva is derived from three of six multipotential vulval precursor cells (VPCs), which receive an inductive signal from the anchor cell (AC) in the somatic gonad (Figure 1) [32]. The VPCs that form the vulva undergo three rounds of division and subsequent morphogenesis. VPCs that do not receive adequate signal from the anchor cell divide only once and become part of a large syncytial epidermis that covers most of the worm. The inductive signal, LIN-3, is an EGF-like growth factor, produced by the anchor cell. LIN-3 activates the LET-23, a *C. elegans* homolog of EGF RTK, in the VPCs [33] [34]. LET-23 activation initiates a signaling cascade that involves downstream effectors such as SEM-5, SOS-1, LET-60, LIN-45, MEK-2 and SUR-1/MPK-1 which are the nematode homologs of the mammalian Grb2 adaptor, the mSOS guanine nucleotide exchange factor, the Ras GTP-binding protein, the Raf serine/threonine kinase, the MAPK kinase and the MAPK, respectively [35] [36] [37] [38] [39] [40] [41] [42] [43] [44] [Chapter 2]. *Loss-of-function (lf)* mutations in any of the signaling proteins in the LET-23 RTK pathway result in less than three VPCs undergoing

vulval differentiation, whereas *gain-of-function (gf)* mutations result in more than three VPCs undergoing vulval differentiation.

Apart from the main RAS-dependent signaling pathway, there are two accessory proteins identified in a screen for the extragenic suppressors of the G13E-activated mutant *let-60 ras*. *ksr-1*, which encodes a conserved Raf-related serine/threonine kinase, has been shown to act downstream of or in parallel to *let-60 ras* and upstream of *lin-1*, a putative ETS transcription factor [45] [46]. *sur-8/soc-2*, which encodes a conserved Ras-binding protein with leucine-rich repeats, appears to act downstream of or in parallel to *let-60 ras* but upstream of *lin-45 raf* [47] [48]. Mutations in either loci do not affect normal vulval induction. However, a *sur-8/soc-2* mutation significantly synergizes with a *loss-of-function* mutation in *ksr-1*, suggesting that they are collectively essential in the presence of the main RAS-dependent signaling pathway.

A mutant of *ptp-2*, another positive effector for *let-23*-mediated vulval signaling, identified by a PCR-based screen for transposon-mediated deletion, appears not to affect vulval induction [49]. SH2 domain-containing PTP-2 has a predicted protein structure similar to the mammalian SHPs and *Drosophila* Corkscrew. *Loss-of function* in *ptp-2* suppresses the multiple vulva (Muv) phenotype induced by a *loss-of-function lin-15* mutation, and an activated *let-23 EGFR* or *let-60 ras* mutation. The role of *ptp-2* function in the vulval signaling pathway is not clear.

A *sos-1*-independent signaling activity for *ras* activation is suggested since animals removing *lin-3* ligand shows more severe phenotype than those removing sos-1 [Chapter 2]. This parallel pathway appears to be independent of *sli-1* regulation, *ptp-2* signal transduction, and can not be simply explained by the relinquishment of the inhibition from *gap-1*.

Three negative regulators of LET-23 mediated vulval differentiation were isolated as extragenic suppressors of *let-23(lf)* and have been cloned. UNC-101 is a *C. elegans* homolog of mammalian AP-47 clathrin medium chain protein [50]. SLI-1 is a *C. elegans* homolog of mammalian proto-oncoprotein c-Cbl [51] [52] [Chapter 3]. GAP-1 is a *C. elegans* homolog of mammalian Ras-GTPase activating protein (RasGAP) [53]. One negative regulator was isolated as an extragenic enhancer of *sli-1(lf)* and has also been cloned. ARK-1 is a non-receptor protein tyrosine kinase (PTK) related to the Ack sub-family [54]. Another negative regulator was identified by a comparative analysis of RTK pathways with similar signaling logic. CLR-1 is a *C. elegans* homolog of *Drosophila* PTP69D receptor tyrosine phosphatase [Chapter 4]. Mutations in each of these five negative regulators are silent by themselves. However, in combination with mutations in any other loci defining negative regulators results in a Muv phenotype (G. Jongeward, N. Hopper, C. Chang, C. Lacenere and P. Sternberg, unpublished).

Yet another set of apparently redundant negative regulators are known. Genetically, there are two classes of the so called synthetic multivulva (synMuv) genes, class A and class B. Class A genes include *lin-8*, *lin-15*A, *lin-38*, and *lin-56* and class B genes include *lin-9*, *lin-15*B, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55*, and *tam-1* [55] [56] [57] [Appendix 1]. Animals contained mutations in only class A or class B genes are wild-type, whereas animals carrying mutations in both classes of genes are Muv. Two class B synMuv genes, *lin-35* and *lin-53*, have been shown to encode proteins similar to Rb and its binding protein RbAp48. *lin-15*, a complex locus containing two mutable genetic activities (A and B), encodes two non-overlapping transcripts involved in the negative regulation of vulval induction [58, 59]. The predicted proteins from *lin-15*A and *lin-15*B transcripts are not similar to any identified proteins. Mutations in either *lin-15*A or *lin-15*B are phenotypically wild-type and a Muv phenotype can be

induced only if both activities are removed. There seems to be a fundamental difference between synMuv genes and the negative regulators discussed above. The negative effects of class A synMuv genes are strictly redundant to class B synMuv genes, whereas the negative effects of those negative regulators mentioned before are partially redundant among them (Figure 2). It is unclear how, mechanistically, the synMuv proteins regulate LET-23-Ras signaling.

Recently, another negative regulator, *sur-5*, was identified in a screen for the extragenic suppressors of a dominant negative *(dn) let-60 ras* allele [60]. *sur-5* encodes a novel protein with one potential ATP or GTP binding motif and two potential AMP binding motifs. Mutations in *sur-5* have no obvious phenotypes of their own and does not suppress *loss-of function* mutations in the *let-23 EGFR* signaling pathway either upstream or downstream of *let-60 ras*, including *let-60 ras* itself. It is interesting that *sur-5* mutations specifically suppress one of two subsets of *let-60 ras (dn)* alleles. Since all the *let-60 ras (dn)* mutations are suppressed by the *let-60 ras (gf)* mutation in trans-heterozygotes [35] [36] [61], it is believed that the toxic effect of a dominant negative LET-60 RAS protein is due to the competition with wild-type LET-60 RAS protein for the upstream guanine nucleotide exchange factor (GNEF) instead of downstream effectors. These results raise the possibility that there might be at least two different activators for LET-60 RAS and only one of them is titratable by the subset of *let-60 ras (dn)* mutant proteins that is suppressed by the *sur-5* mutations. The existence of another RasGEF for RAS is also implied in the our genetic studies of *sos-1* mutants [Chapter 2].

EGFR signaling in ovulation

LET-23 function in the somatic gonad is required for ovulation in hermaphrodites and is independent of RAS. While mutations in *lfe-1/itr-1* and *lfe-2* suppress the ovulation defects associated with *let-23(lf)* mutant, mutations in the components of RAS pathway do not [29]. *lfe-1/itr-1* encodes a *C. elegans* inositol (1,4,5) trisphosphate receptor (IP3R) and *lfe-2* encodes an inositol (1,4,5) trisphosphate-3-kinase (IP3K). Since a *gf* mutation in *lfe-1/itr-1* and a *lf* mutation in *lfe-2* rescue the ovulation defects of a *let-23(lf)* mutant, *lfe-1/itr-1* is inferred to be a positive effector of LET-23 signaling whereas *lfe-2* a negative regulator. The comparison of *C. elegans* vulval development and hermaphrodite ovulation thus provides an excellent example of how a RTK evokes its tissue specificity by deploying the distinct signaling cascades. The *let-23(sy97)* mutant animals are defective in all *let-23*-mediated phenotypes except for the hermaphrodite ovulation [62]. This mutation deletes the last 56 amino acids of the receptor which include the only three tyrosines (pTyr site 6, 7 and 8) in the receptor carboxy terminal tail which, if phosphorylated, would create SH2 binding sites matching the consensus binding site for the SEM-5 SH2 domain. Consistent with these findings, in the *let-23(null)* background, none of the site deleted by the *let-23(sy97)* mutation is able to confer fertility and *let-23* construct bearing either only site 4 or site 5 confers full or 15% fertility [63].

In *C. elegans* hermaphrodites, there are two developmental events affecting fertility, germline meiotic cell cycle progression and ovulation. While hermaphrodite ovulation is independent of *let-60 ras* activity, germline meiotic cell cycle progression is not. Although *let-60/mek-2/mpk-1* cascade responses to *let-23* activation in most of tissues, the inductive signal and cognate receptor used for *let-60/mek-2/mpk-1*-mediated germline meiotic cell cycle progression is still unknown.

Developmental logic of EGFR pathway - Reciprocal signaling

lin-3 EGF is first produced by the gonadal AC to induce VPCs to divide and differentiate into vulval tissue. After vulval induction, *lin-3 EGF* is also expressed in the 1° vulval lineage (Figure 3) [31]. EGF signaling from cells of the 1° vulval lineages to a subset of ventral uterine cells of the gonad is required for the specification of uterine uv1 cells, which form part of the uterine-vulval connection [Chapter 5]. While mutation of SLI-1 restores most of the reduced LET-23 signaling in the vulva caused by *let-23(lf)* mutation, the defects in uv1 cell fate are only partially rescued. This specificity most likely comes from the quantitative difference of *sli-1* usage. In contrast, a *gap-1(lf)* mutation suppresses the vulval but not the uv1 defect displayed by the same *let-23(lf)* mutation, suggesting that LET-23-mediated signaling is not regulated by GAP-1 in the presumptive uv1 cells. This developmental logic of EGF signaling could provide a basis to understand a universal mechanism for organogenesis.

Table 1 shows genes of *let-23 EGFR* signaling pathway identified in *C. elegans* either by sequence homology search or by genetic mutation. Many sequence homology searches are based on information obtained from the biochemical studies in mammalian systems where a putative role in EGF RTK signaling was implied. Some of the SH2 and PTB domains of signaling molecules, although found to be associated with the activated EGF RTK, are not formally demonstrated regarding their functional relevance in the regulation of signaling (effectors or regulators). For those having a corresponding mutant, the phenotypes observed obviously display their physiological involvement in EGF RTK signaling. Mutants may be recovered by either a classical genetic screen or by a PCR-based screen for deletions. Double-stranded RNA interference is also used to identify some of the signaling components or to confirm the mutant

phenotypes.

EGFR signaling is conserved during evolution

Although the cellular interpretation of signaling are context-dependent, the RAS-dependent EGF RTK signaling pathways are well conserved at the level of their mechanistic aspects during metazoan evolution. As a result, a core set of players required for the execution of signaling share high similarity among species in terms of their functions, protein structures, and one-dimensional amino acid sequences. Differences obtained from evolution predominantly reside in the redundancy of signal transduction and the complexity of its regulation. In *C. elegans*, all known effects of LIN-3 are mediated through LET-23 and there is no evidence that LET-23 has additional ligands apart from LIN-3. In the VPCs, upon the binding by LIN-3, LET-23 is presumably autophosphorylated and that creates docking sites for the proteins containing SH2 or PTB domains, such as SEM-5. Based on results with the mammalian proteins, SOS-1 likely constitutively binds SEM-5 via its proline-rich domain. The activated receptor-bound SEM-5 therefore brings SOS-1 close to the membrane-anchored LET-60 and that catalyzes the release of GDP from LET-60 and allows the loading of GTP onto it.

Mechanisms of negative regulation of LET-23 RTK signaling

Sos/Ras/Raf/MAP kinase pathway, now known to mediate signaling by most if not all RTKs has become a major target for cancer therapeutics as excess signaling through the Ras had previously been linked to tumorigenesis. Unlike the intensively studied mechanistic aspects of signaling transduction by Ras-dependent RTKs, the molecular mechanisms of their negative regulation are still

largely unclear. Studies in the negative regulation of LET-23 RTK pathway thus provide valuable lessons to signal control and therapeutic strategies. Generally, there are two ways to negatively regulate signaling. One is to regulate the basal activity of signaling in the absence of the ligand. The other is to attenuate or desensitize the signaling after activation by the ligand. RasGAP inhibits the Ras activity by stimulating its intrinsic GTPase activity, thereby increasing the level of GDP-bound, inactive Ras [64]. Apart from the catalytic domain, *C. elegans* GAP-1 does not display any significant similarity to other proteins in the database [53]. SH2 and SH3 domains, that are found in the mammalian p120 RasGAP, are not present in the *C. elegans* GAP-1. *C. elegans gap-1* acts to inhibit the vulval induction since *loss-of-function* mutation in the *gap-1* suppresses the vulvaless (Vul) phenotype associated with *loss-of-function* mutations in the *let-23 EGFR/let-60 ras* mediated signaling pathway. It remains an open question whether *C. elegans* GAP-1 is a constitutive inhibitor of RAS and not dependent upon the ligand/receptor.

sli-1 has been shown to inhibit the *let-23 EGFR*-mediated vulval signaling upstream of *let-60 ras* by genetic suppression analysis [51]. So far, there is no report from biochemical studies showing an interaction between c-Cbl and mSOS, but many reports demonstrated the physical association between Cbl/SLI-1 and Grb2/SEM-5 or c-Cbl and activated EGFR [Chapter 3]. c-Cbl binds to Grb2 through its polyproline motifs and colocalizes with EGFR via its diverse SH2 domain, which is dependent upon ligand activation. The sequence of D-Cbl, a *Drosophila* homolog of c-Cbl, ends immediately C-terminal to the RING finger motif [17] [65]. Athough D-Cbl lacks polyproline motifs, expression of the *sevenless* enhancer-driven D-Cbl significantly abolishes the development of R7 photoreceptor neuron, suggesting that polyproline motifs might be evolutionarily dispensable for the function of c-Cbl-like proteins in regulating

the RTK signaling. Although our preliminary data show that the polyproline motifs are not essential for *sli-1* function in the vulva, the presence of polyproline motifs do help to achieve the full activity of *sli-1* [Chapter 3]. Contrary to the results from recent biochemical assays in mammalian systems that ubiquitin ligase activity of RING finger motif is responsible for the negative effect of Cbl, the genetic evidences in *C. elegans* suggests a RING finger-independent regulation by SLI-1 [Chapter 3].

Our current models for *sli-1* functions propose two roles. The major role of *sli-1* might be to attenuate signaling after activation has occurred. This model is supported by the evidence that c-Cbl-like proteins only associate with EGFR in an activation-dependent manner. Indeed, by analyzing the systematically mutagenized *let-23* constructs containing substitutions in the carboxyl-terminal tyrosine residues, we have identified one inhibitory phosphotyrosine residue, which can overcome the negative regulation by *sli-1* when it is mutated [Chapter 3]. The observation is strengthened by a recent solved crystallographic structure of c-Cbl complexed to a tyrosine-phosphorylated inhibitory site of protein tyrosine kinase ZAP-70 [66]. The interaction is mediated by a divergent SH2 domain of c-Cbl, which is conserved in SLI-1, and a phosphotyrosine of ZAP-70, which is in a similar amino acid context to the one in LET-23 we identified. The minor role of *sli-1* might be to regulate the basal activity of signaling in quiescent state by competing with SOS-1 for the binding of SEM-5, thereby decreasing the chance that the spontaneously activated receptor recruits the SOS-1-bound SEM-5 in the absence of ligand. Upon stimulation by the inductive signal, SLI-1 molecules might switch to bind the tyrosine-phosphorylated receptors, thereby relinquishing the interference on the binding between SOS-1 and SEM-5. While *sli-1* negatively regulates the *ras*-dependent vulval differentiation, it does not affect the *lfe-1*-dependent hermaphrodite ovulation. The simplest interpretation

is that there is a tissue-specific expression or activation of *sli-1*. Alternatively, the *ras*-dependent and *lfe-1* dependent signaling cascades might have fundamental differences such that *sli-1* can only inhibit one signaling cascade but not the other. For example, *ras*-dependent and *lfe-1* dependent signaling cascades are elicited by distinct phosphotyrosine residues of *let-23 EGFR*, which could have different steric orientations. While the activated receptor-bound *sli-1* is able to execute its negative regulation in one steric orientation, it fails in the other.

A localized *let-23 EGFR*-dependent negative regulation has been proposed previously since a *reduction-of-function* mutation in *let-23 EGFR*, which results in the mislocalized receptor, causes a Muv phenotype in a *sli-1* or *gap-1* mutant background [51] (G. Jongeward and P. Sternberg, unpublished). Genetic mosaic analysis showed that the presence of *let-23 EGFR* in the proximal VPCs inhibits the vulval induction in their more AC-distal VPCs [53]. A possible mechanism for this observation is that the AC-proximal VPCs bind and sequester the AC-produced ligand LIN-3 EGF, thereby preventing the ligand from reaching more distal VPCs. In this view, the sequestering mechanism serves to down regulate the ligand after it activates the receptor in the AC-proximal VPCs.

The negative regulation of *let-23 EGFR*-mediated vulval signaling by synMuv genes is ligand-independent: gonad ablation does not affect the Muv phenotype induced in the *lin-15*AB mutant background [59] [67]. Mutations in *let-23 EGFR* and its downstream effectors suppress the Muv phenotype induced by *lin-15* mutations. Taken together, these data suggest *lin-15* acts upstream of *let-23 EGFR* but downstream of or in parallel to *lin-3 EGF*. Although mosaic analysis suggests that *lin-15* functions in the hyp7 epidermis and hence regulates *let-23* signaling in the VPCs in a cell non-autonomous manner [68], the LIN-15 proteins are present in the nuclei of the VPCs (L. Huang, J. DeModena and P. Sternberg, unpublished). Indeed, *lin-35* Rb and *lin-53* p48, two loci previously

assigned to the class B synMuv pathway, have been shown to express in the VPCs nuclei [57]. If *lin-15* does function in the nuclei of the VPCs to negatively regulate the *let-23*-mediated vulval signaling as opposed to its downstream nuclear targets, there are two possible targets for *lin-15* action, LET-23 EGFR and its activators. It is less likely that *lin-15* only acts to inhibit the expression of *let-23* since overexpression of *let-23* does not cause a Muv phenotype as does by *lin-15* mutations [33] (G. Lesa and P. Sternberg, unpublished). The combinatorial effects on the expression of *let-23* and its activators might, ultimately, explain *lin-15* function in the VPCs.

Conclusions

C. elegans LET-23-mediated vulval induction requires *C. elegans* homologs of Egf, Egfr, Grb-2, Sos, Ras, Raf, Mapk kinase, and Mapk, which are conserved during metazoan evolution. New positive effectors and negative regulators of EGFR-mediated signaling have been identified by *C. elegans* genetic screens, sequence homology searches, and comparative analyses of RTK pathways with similar signaling logic. One way in which EGFR has tissue-specificity is to use distinct signaling cascades in different tissues. The developmental logic of EGFR signaling discovered in *C. elegans* can provide a basis for studying the universal mechanism for organogenesis.

Key unanswered questions

One way to modulate the strength of signaling is by controlling the production and presentation of the ligand. Identification of tissue-specific transcriptional regulator(s) and protease(s) will be required to understand this signaling

regulation. RAS activity is necessary not only for the vulval induction but also for the subsequent patterning events [31] (C. Chang and P. Sternberg, unpublished). The amplitude and duration of RAS-dependent EGFR signaling required for proper vulval development is unclear. To address such a question, it is essential to develop an assay to monitor the signaling activity during animal development. Although binding to the EGFR seems to be necessary for the negative regulation by *sli-1*, the mechanisms by which *sli-1* exerts its negative effects are not well understood. Ubiquitin ligase activity of RING finger motif only explains partly for regulation by SLI-1. Since *sli-1* mutation fails to complement a *gap-1* mutation (C. Chang, unpublished), *sli-1* and *gap-1* might act in a linear pathway where *sli-1* exerts its negative effects by directly or indirectly activating *gap-1*. This hypothesis is supported by the only weak synergy between *sli-1* and *gap-1* mutants [Chapter 3]. How does SOC-2/SUR-8 fit into the RAS signaling? If interaction between SOC-2/SUR-8 and RAS is necessary for the normal function of signaling, SOC-2/SUR-8 could be either another effector for RAS or a modulator to facilitate the association between RAS and effectors. PTP-2 functions as a supporting component during vulval development. How is it integrated into the main signaling framework? The identification of PTP-2 substrates should allow us to understand its contributions to the signaling.

In the mammalian model system, RTKs-mediated signaling and G protein-mediated pathway involve a highly cooperative network that sense a large number of cellular inputs and process these information to orchestrate an appropriate set of cellular responses. A recent study shows that the MAPK pathway is important for G protein-coupled receptors (GPCRs)-mediated chemotaxis to a set of odorants in *C. elegans* [69]. The elucidation of the molecular mechanisms whereby GPCRs activate MAPK is believed to be central to understanding how these receptors transmit signals to confer a dynamic

regulatory role for the MAPK cascade in perception in olfactory organs. The potential candidates for linking signaling from GPCRs to MAPK include βγ heterodimers, nonreceptor tyrosine kinase, receptor tyrosine kinase, Ca^{2+}-dependent RasGEF, PI3 kinase and phospholipase C.

One aspect of vulval development not explicitly considered here might prove especially relevant to tumor cell biology. After inducing the vulva, the gonadal anchor cell invades the vulval epithelium, initiating the hole that will ultimately connect the lumen of the uterus to that of the vagina [70] [71] (R. Palmer, D. Sherwood, K. Tietze and P. Sternberg, unpublished). This process appears analogous to tumor cell invasion during metastasis, and might provide a genetic model system to investigate invasion.

In the post-genomic era, the challenges we face are how to create tools for analyzing the complex data derived from the high-throughput function determination and expression profiling of defined sets of signaling molecules, and for producing robust predictions of the stoichiometric and spatial interactions among signaling components. From these, mathematical models and theories of the biochemical and genetic network structure and dynamics can be created. With understanding the basis of a cell's ability to process the complex information it receive from the environment into the robust developmental consequences, we ought to be able to design pharmacological perturbations to redirect abnormal cells to behave in a predictable, designed way.

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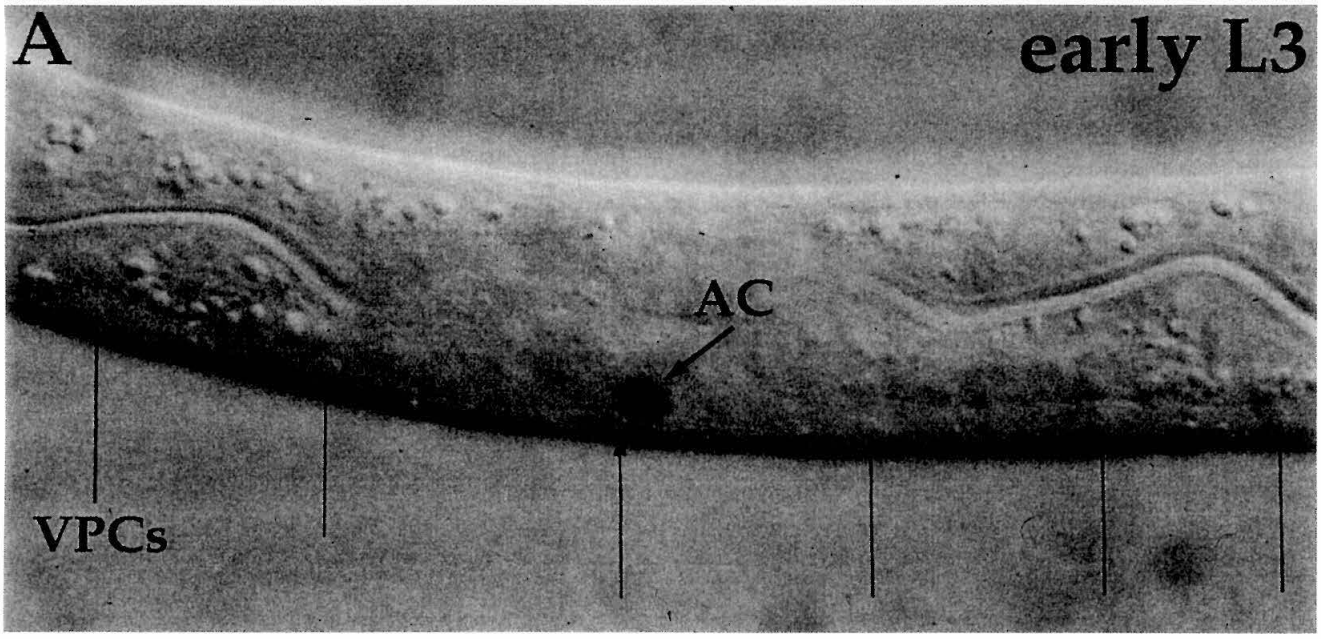
Figure 1

A. *C. elegans* vulva development occurs postembryonically. Six ventral midline-located VPCs are competent to adopt any of three cell fates (1°, 2°and 3°). In the early third larval (L3) stage, upon induced by a gonadal AC-produced signal, the AC-closest VPC adopts 1° fate, which gives rise to eight descendants, while the adjacent VPCs adopt 2° fate, which gives rise to seven descendants. The further away three VPCs adopt 3° nonvulval fate, due to insufficient receiving of the inductive signal.

B. After the last round of cell division, cells of a canonical 1° lineage move dorsally and detach from the ventral cuticle. By the mid-L4 stage, they form a symmetric arch and separate anterio-posteriorly and left-right to create a hole so that eggs can pass through it. The 2° lineage is asymmetric: the proximal cells detach and migrate dorsally and the AC-distal cells keep attaching to the ventral epidermis. By the mid-L4 stage, they form a characteristic structure.


A
early L3
AC
VPCs

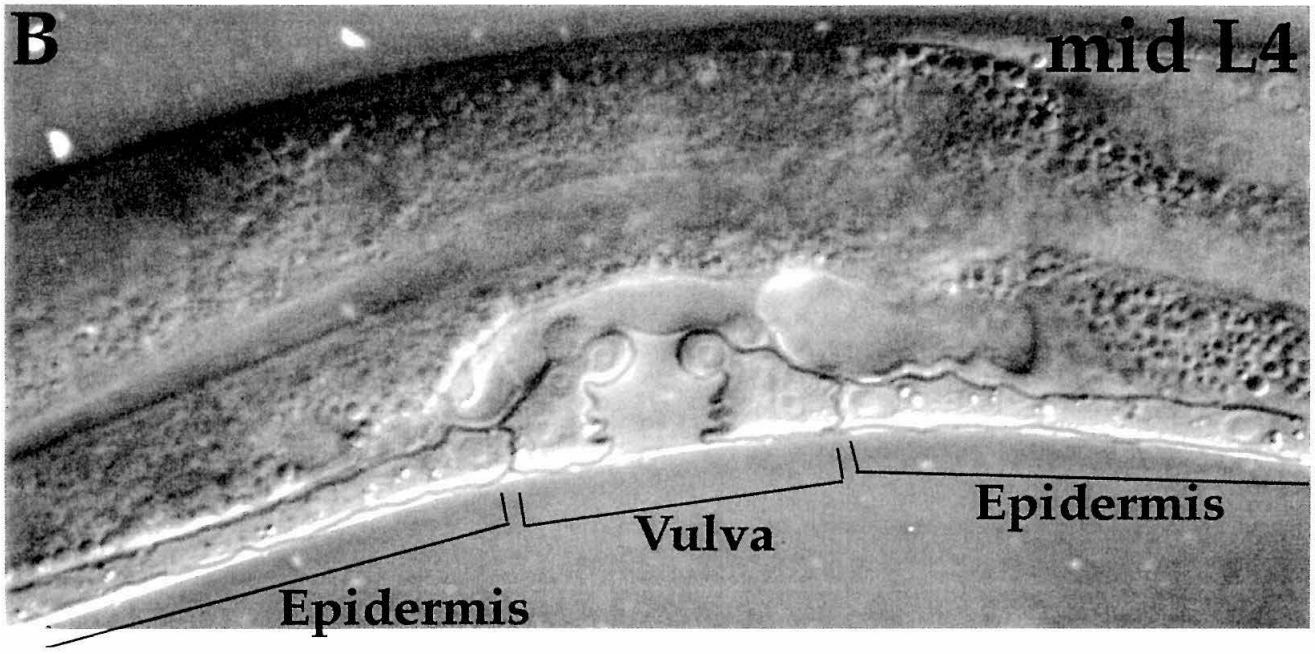

B
mid L4
Epidermis
Vulva
Epidermis

Figure 2

A. A hypothetical regulatory circuit operated by the synMuv genes. Arrows indicate a positive effect and bars indicate a negative effect. There is a strict redundancy between synMuv class A genes and class B genes in negatively regulating the *let-23 EGFR* signaling pathway. Only mutation(s) in class A genes combined with mutation(s) in class B genes will result in the excessive signaling and the Muv phenotype.

B. A hypothetical regulatory circuit operated by *sli-1*, *unc-101*, *rok-1* and *gap-1*. Arrows indicate a positive effect and bars indicate a negative effect with thickness of the line reflects strength of the effect. X, Y and Z represent the negative regulations provided by these signaling regulators. The inhibitory effects performed by any two of them are partial redundant such that mutations in any two of them will lift the restraint on one signaling node, that results in the excessive signaling and the Muv phenotype.

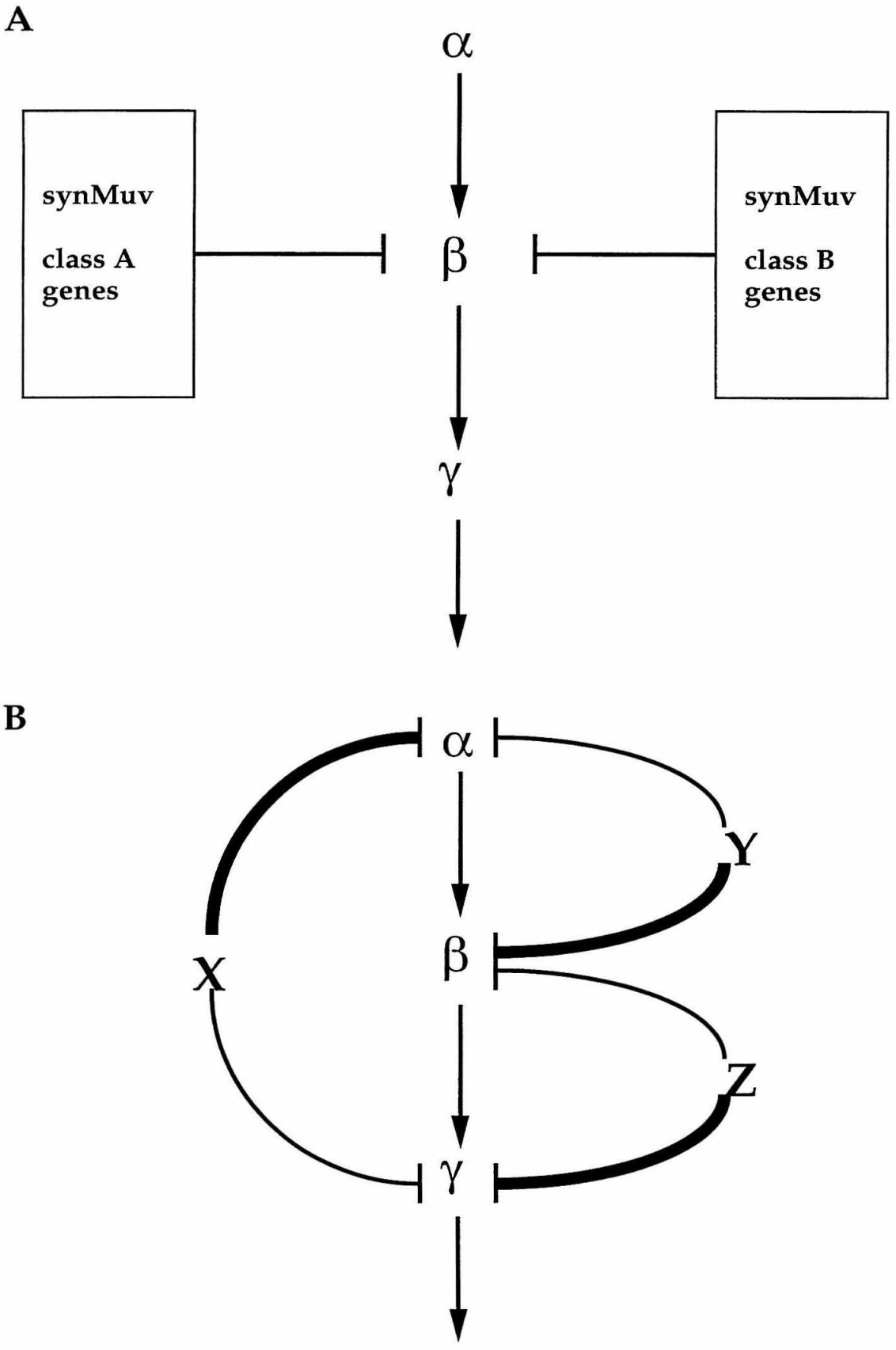

A
α
synMuv
class A
genes
β
synMuv
class B
genes
γ
B
α
Y
X
β
Z
γ

Figure 3

Reciprocal EGF signaling from the vulva specifies the uv1 cell fate.

vulF, the granddaughter of the 1° VPC, is the dorsal-most 1° vulval progeny.

uv1, the ventral-most uterine cell, makes the direct contact with vulF during the development of the uterine-vulval connection.

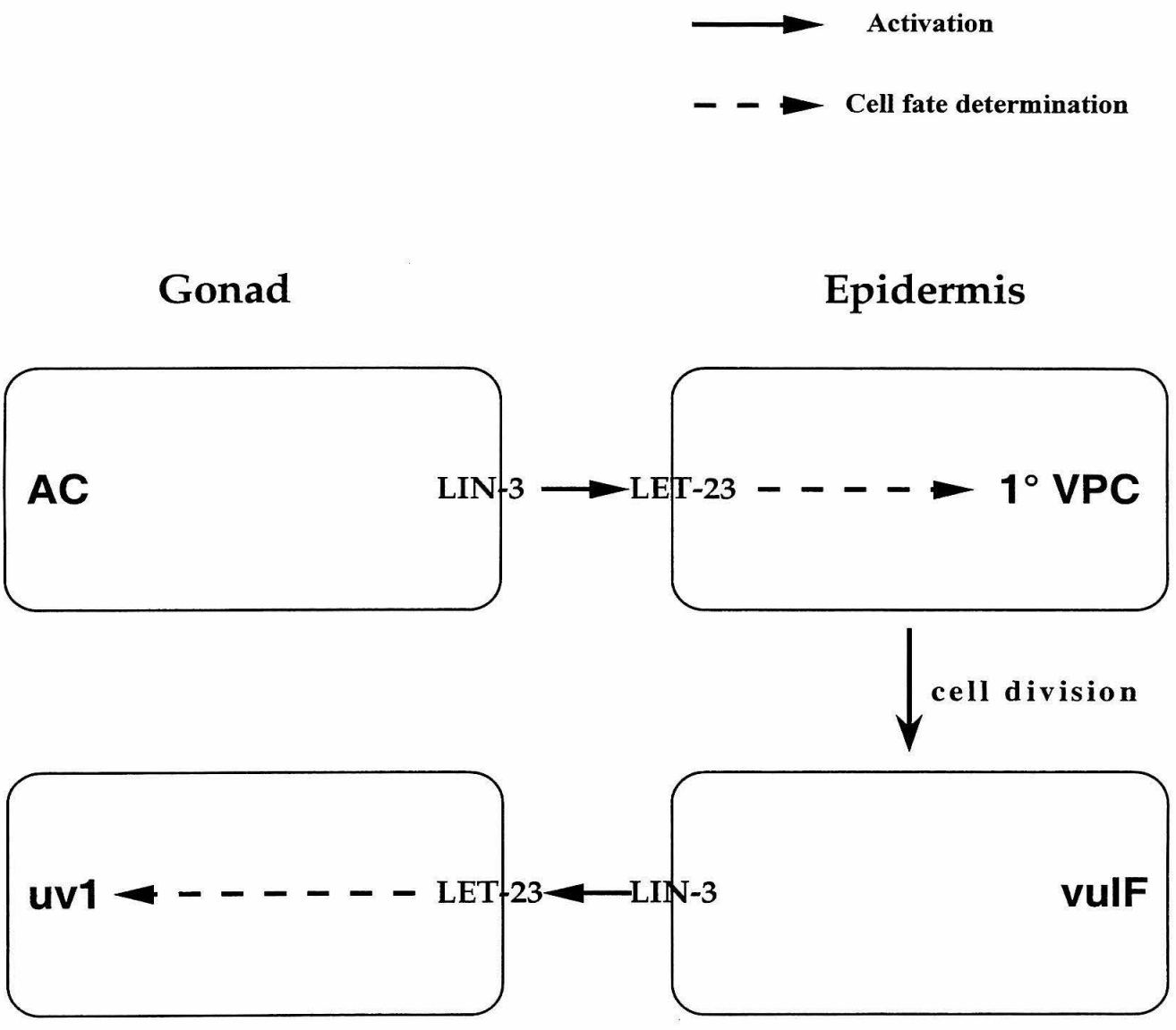

Activation
Cell fate determination
Gonad
Epidermis
AC
LIN-3
LET-23
1° VPC
cell division
uv1
LET-23
LIN-3
vulF

Table 1

Genes involved in *let-23 EGFR*-mediated signaling

Defined by mutants

	Genes	Genetic screens used to isolate the mutant alleles	Available mutations	Linkage groups	Nature of proteins
Ligand	*lin-3*$^{\Delta}$	1,†	*lf,gf*	IV	EGF
Receptor	*let-23*^{i}	1&2,3	*lf,gf*	II	EGFR
Positive effectors in vulva induction	*sem-5*	2&4	*lf*	X	Grb2 adaptor
	sos-1	2&4&5	*lf*	V	RasGEF
	let-60$^{\Delta}$	2&6,3&7,2	*lf,gf,dn*	IV	Ras-like GTPase
	ptp-2	8	*lf*	II	SHPs
	sur-8/soc-2^{i}	4&9	*lf*	IV	leucine-rich repeats
	ksr-1	9	*lf*	X	Raf-related kinase
	lin-45^{i}	2&9,10,10	*lf,gf,dn*	IV	Raf Ser/Thr kinase
	mek-2^{i}	9,10,10	*lf,gf,dn*	I	MAP kinase kinase
	mpk-1/sur-1^{i}	9	*lf*	III	MAP kinase
in ovulation	*lfe-1/itr-1*	11	*gf*	IV	IP3R
Negative regulators in vulva induction	*lin-15*	3	*lf*	X	Novel protein
	sli-1	5&7	*lf*	X	Proto-oncogene c-Cbl
	ark-1	12	*lf*	IV	Non-receptor PTK
	unc-101	7	*lf*	I	AP47
	gap-1	7	*lf*	X	RasGAP
	clr-1	13	*lf*	II	RTP
	sur-5	14	*lf*	X	Novel protein
in ovulation	*lfe-2*	11	*lf*	I	IP3K

Defined by sequence similarity

Genes	Nature of protein
Dos	Daughter of sevenless
PLCγ	Phospholipase Cγ
Nck	SH2/SH3 adaptor
Crk	SH2/SH3 adaptor
PI3K p110	Catalytic subunit of PI3 kinase
PI3K p85	Regulatory subunit of PI3 kinase
Cnk	Connector enhancer of KSR

1. Direct screening for the Vul mutants from the wild-type animals.
2. Extragenic suppressors of the Muv phenotype of *lin-15(lf)* mutants.
3. Direct screening for the Muv mutants from the wild-type animals.
4. Extragenic suppressors of the "Clear" phenotype of *clr-1(lf)* mutants. *clr-1* encodes a *C. elegans* homolog of receptor tyrosine phosphatase. "Clear" phenotype indicates *the clarity of* cell boundaries, reflecting a hyperactive FGF receptor signaling [38].
5. Double-stranded RNA interference.
6. Intragenic revertants of *let-60(dn)* mutants.
7. Extragenic suppressors of the Vul phenotype of *let-23(lf)* mutants.
8. PCR-based screening for transposon-mediated deletion mutant.
9. Extragenic suppressors of the Muv phenotype of G13E-activated *let-60 ras* mutants.
10. Mutations corresponding to either mammalian or *Drosophila* changes were created by site-directed mutagenesis and the transgenes are put under control of the heat shock promoter.
11. Extragenic suppressors of the sterility phenotype of *lin-3(lf)* mutants.

12. Extragenic enhancers of the "pre-Muv" phenotype of *sli-1(lf)* mutants. "*pre-Muv*" represents a state where an excessive *let-23 EGFR* signaling does not overcome the threshold to cause a Muv phenotype but is sensitive to the fluctuation of the signaling.
13. Extragenic suppressors of the scrawny body morphology of *egl-15(lf)* mutants.
14. Extragenic suppressors of the Vul phenotype of *let-60(dn)* mutants.

† When a genetic *gain-of-function* mutation is not available, increase of gene dosage by the wild-type transgene mimics the *gain-of-function* phenotype. To give an effect by overexpressing transgene, the endogenously expressed-gene products must be relatively limited when compare to other proteins.

Δ Multiple copies of transgenes produce the dominant Muv phenotype, suggesting that the quantities of their gene products are normally limited in the vulval signaling.

ⁱ Multiple copies of transgenes do not produce the dominant Muv phenotype, suggesting that the quantities of their gene products are not normally limited in the vulval signaling.

Chapter 2

Caenorhabditis elegans SOS-1 is necessary for multiple RAS-mediated developmental signals

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Abstract

Vulval induction in *C. elegans* has helped define an evolutionarily conserved signal transduction pathway from receptor tyrosine kinases (RTKs) through the adaptor protein SEM-5 to RAS. One component present in other organisms, a guanine nucleotide exchange factor for Ras, has been missing in *C. elegans*. To understand the regulation of this pathway it is crucial to have all positive-acting components in hand. Here we describe the identification, cloning and genetic characterization of *C. elegans* SOS-1, a putative guanine nucleotide exchanger for LET-60 RAS. RNA interference experiments suggest that SOS-1 participates in RAS dependent signaling events downstream of LET-23 EGFR, EGL-15 FGFR and an unknown RTK. We demonstrate that the previously identified *let-341* gene encodes SOS-1. Analyzing vulval development in a *let-341* null mutant, we find a SOS-1-independent pathway involved in the activation of RAS signaling. This SOS-1-independent signaling is not inhibited by SLI-1/Cbl and is not mediated by PTP-2/SHP, raising the possibility that there could be another RasGEF.

Introduction

Polypeptide hormones bind cell surface receptors containing tyrosine kinase activity to regulate cell proliferation, differentiation, migration and metabolism. All receptor tyrosine kinases exhibit a similar molecular architecture and apparently are activated by a common mechanism (Gutch *et al.*, 1998). Ligand binding to the extracellular domain induces receptor dimerization which in turn leads to activation of the catalytic kinase domain (Lemmon and Schlessinger, 1994). Autophosphorylation of tyrosine residues that are located in the C-terminal region leads to the physical recruitment of cytoplasmic signaling molecules with phosphotyrosine (pTyr)-recognition modules, such as SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains (Pawson, 1995). These receptor-binding proteins include the adapter Grb2, which leads to Ras activation, Ras-GTPase activating protein, phospholipase C-(PLC)-γ, the tyrosine phosphatase SHP-2, the regulatory subunit of phosphatidylinositol-3-OH kinase (Heldin, 1995; Pawson, 1995; Ullrich and Schlessinger, 1990), the scaffolding protein Shc (Pelicci *et al.*, 1992), and the proto-oncoprotein c-Cbl (*Meisner et al.*, 1997). The association between proteins with SH2/PTB domains and receptor pTyrs serves as the initial step in the recruitment of an activated RTK signaling complex. Different SH2/PTB domains have distinct specificities for the pTyr residues dependent on the surrounding amino acid residues (Kavanaugh *et al.*, 1995; Kavanaugh and Williams, 1994; Panayotou and Waterfield, 1993; Songyang *et al.*, 1993; Songyang *et al.*, 1994). Association of RTKs with substrates is therefore dependent upon the amino acid context of the pTyr, and that, in part, determines which proteins join the signaling complex and what cellular responses are evoked (Pawson and Saxton, 1999; Schlessinger and Ullrich, 1992). The achievement of a precise amplitude and duration of the signaling required

for the reproducible developmental consequences observed can be obtained by regulating the production of signaling molecules and their presentation to their substrates. Excessive or prolonged RTK signaling has been implicated in many cases of malignant transformation (Alroy and Yarden, 1997; Cheng *et al.*, 1998). Therefore, proper mechanisms must be employed to strongly control the signaling from RTKs to prevent spontaneous, ligand-independent activation and downregulate signaling after it has been activated.

EGF RTK signaling is used multiple times during the normal development of *C. elegans*, mediating vulval induction, viability, male spicule development, hermaphrodite ovulation, and differentiation of the ventral uterus and posterior ectoderm (Aroian and Sternberg, 1991; Chamberlin and Sternberg, 1994; Chang *et al.*, 1999; Clandinin *et al.*, 1998; Ferguson and Horvitz, 1985; Jiang and Sternberg, 1998). The high resolution of the vulval induction assay makes it very sensitive, allowing one to identify mutants and to reflect the relative strength of RAS-dependent signaling (Han and Sternberg, 1991). All the other developmental events are also RAS-dependent, with the exception of hermaphrodite ovulation, which is instead mediated through an inositol trisphosphate signaling pathway (Clandinin *et al.*, 1998).

The wild-type vulva is derived from three of six multipotential vulval precursor cells (VPCs), in response to an inductive signal from the anchor cell (AC) in the somatic gonad (Horvitz and Sternberg, 1991). The induced VPCs undergo three rounds of division and subsequent morphogenesis to form the vulva. VPCs that do not receive adequate signal from the anchor cell divide only once and become part of the worm's large syncytial epidermis. The inductive signal, LIN-3, is an EGF-like growth factor produced by the anchor cell. LIN-3 activates LET-23, a *C. elegans* homolog of EGF RTK, in the VPCs (Aroian *et al.*, 1990; Hill and Sternberg, 1992). LET-23 activation initiates a signaling cascade

that involves downstream effectors such as SEM-5, LET-60, LIN-45, MEK-2 and SUR-1/MPK-1 which are the nematode counterparts of the mammalian Grb2 adaptor, the Ras GTP-binding protein, the Raf serine/threonine kinase, the MAPK kinase and the MAPK, respectively (Beitel *et al.*, 1990; Clark *et al.*, 1992a; Han *et al.*, 1993; Han and Sternberg, 1990; Kornfeld *et al.*, 1995; Lackner *et al.*, 1994; Wu and Han, 1994; Wu *et al.*, 1995). *Loss-of-function (lf)* mutations in these signaling proteins result in less than three VPCs undergoing vulval differentiation, whereas *gain-of-function (gf)* mutations result in more than three VPCs undergoing vulval differentiation.

In addition to the main RAS-dependent signaling pathway, several accessory proteins have been identified. *ptp-2*, which encodes a SH2 domain-containing protein similar to the mammalian SHPs and *Drosophila* Corkscrew, appears to affect vulval induction only in a sensitized genetic background and behaves as a positive effector of the LET-23 pathway (Gutch *et al.*, 1998). Loss-of function in *ptp-2* suppresses the multiple vulva (Muv) phenotype induced by a loss-of-function *lin-15* mutation, and an activated *let-23* or *let-60* mutation. *lin-15* encodes two novel proteins that somehow inhibit Ras-dependent vulval signaling, and *lin-15* mutation formally acts as if LET-23 was activated in a ligand-independent manner (Clark *et al.*, 1994; Huang *et al.*, 1994).

FGFR is another RTK whose developmental roles are dependent upon RAS. Genes involved in the FGFR pathway were defined by mutations affecting sex myoblast migration, including EGL-17 FGF, EGL-15, SEM-5, and several RAS-MAP kinase cascade components (Chen *et al.*, 1997; Clark *et al.*, 1992a; Stern and Horvitz, 1991; Sundaram *et al.*, 1996). Others, including *soc-1* and *soc-2*, were defined by their ability to suppress a clear (Clr) phenotype, which appears to be caused by excess EGL-15 signaling (Selfors *et al.*, 1998).

Mutations in *let-60/mek-2/mpk-1* cause a delay in pachytene exit during meiosis resulting in sterility (Church *et al.*, 1995). The inductive signal, cognate receptor, and their signal transducers used for *let-60/mek-2/mpk-1*-mediated germline meiotic cell cycle progression are still unknown (Sternberg and Han, 1998).

Ras-dependent RTK signaling pathways are well conserved at the level of their mechanistic aspects throughout metazoan evolution. Ras is activated following the recruitment of Sos to the plasma membrane, which stimulates guanine nucleotide exchange. Recruitment of Sos follows receptor activation and is dependent upon the adaptor protein Grb2. Sos contains two structural domains, CDC25 and DH, which act as exchange factors specifically upon the closely related Ras and Rho family GTPases, respectively (Chardin *et al.*, 1993; Nimnual *et al.*, 1998). An EGF RTK substrate, Eps8, and its associated protein, E3b1, have been shown recently to regulate the transduction of signals from Ras to Rac through Sos (Scita *et al.*, 1999). Despite our understanding of Sos function, obtained primarily from work on flies and mammals, the identification of the exchange factor for RAS in *C. elegans* has remained elusive. For our goal of understanding how receptor tyrosine kinase signaling is regulated, it is necessary to identify all the positive and negative acting components. To this end we cloned the previously unidentified *C. elegans* Sos homolog, *sos-1*, and show that it is encoded by the *let-341* gene. We demonstrate SOS-1 activity is required for normal RAS activation. In addition to a requirement for SOS-1, we show that there is a SOS-1 independent pathway operating in vulval induction. This SOS-1 independent pathway does not utilize PTP-2. This raises the possibility that there remain other activators of RAS signaling that are yet to be identified.

Results

Isolation of a Sos homolog in C. elegans

The *C. elegans* Sequencing Consortium (1998) identified an incomplete genomic sequence, which GeneFinder predicts to encode a *C. elegans* homolog of Sos. By using reverse transcription-PCR (RT-PCR) and 5′ and 3′ rapid amplification of cDNA ends (RACE), we have been able to obtain a nearly complete cDNA of this gene and confirm that it encodes a Sos homolog (Figure 1). We will refer to this gene as *sos-1* (for son of sevenless homolog). A SL1 trans-spliced leader sequence, which is immediately followed by the putative initiation methionine, has been identified in the 5′ end of the cDNA. However the cDNA is lacking the extreme 3′ sequence as the termination codon is still missing. This is due to a highly adenylated sequence in the 3′ terminus which presumably prevents the recovery of the 3′ end by use of a poly(dT) primer (Figure 1A). The longest cDNA fragment was sequenced and this 4.2-kb cDNA contains a single long open reading frame (ORF) (GenBank accession number AF251308). We obtained two shorter splice variants predicted to encode proteins that end prematurely immediately after exon 12 and 13 (see materials and methods). Sequence analysis of the corresponding genomic region encompassing this 4.2-kb cDNA revealed that the coding region of this gene spans a region over 30.6 kb and includes a long 13.6-kb intron. Conceptual translation of the 4.2-kb ORF predicted a protein of 1,413 amino acids with signature motifs that match the guanine nucleotide exchange factor for Ras. The predicted structure of SOS-1 contains one Dbl homology (DH) domain followed by one pleckstrin homology domain at the amino terminus and a carboxyl-terminal CDC25 catalytic domain followed by

two PxxP motifs (Figure 1B). CDC25 domain is the most conserved region in RAS-binding exchange factors among species (Figure 1C).

Phenotypic effects displayed by the progeny of sos-1 dsRNA-injected hermaphrodites

To understand the biological functions of *sos-1*, we used double-stranded RNA interference (RNAi) to transiently knock out gene expression during animal development. RNAi has been shown to robustly and efficiently interfere, if not remove, gene expression, in many model systems including *C. elegans*, *Drosophila*, *Trypanosoma brucei*, and plants (Fire *et al.*, 1998; Sharp, 1999). A 1.6-kb *sos-1* cDNA, which represents the sequences encoding the C-terminal portion of the DH and the entire PH domains, was subcloned into the pCRII-TOPO vector. The nucleotide sequence of this 1.6-kb cDNA is not similar to any other gene identified by the *C. elegans* sequencing consortium (as of Apr. 2000). Sense and antisense RNA were synthesized from T7 and Sp6 promoters, respectively, and sense/antisense annealing was carried out *in vitro* prior to microinjection.

RNAi reveals that SOS-1 participates in those functions mediated by LET-60 RAS. Progeny of hermaphrodites injected with *sos-1* dsRNA showed a low penetrance of L1-L2 lethality (8%; n=66). Animals escaping this early larval lethality generally displayed slow growth and a scrawny body morphology, resembling the phenotype of hypomorphic *egl-15* mutants, an FGFR homolog (61/61 animals examined) (Figure 4B). When examined under Nomarski optics, uterine cells are generally mis-positioned or mis-specified (data not shown). During oogenesis, a significant retardation of pachytene exit is observed (11/11 animals examined) (Figure 3D). By young adult stage, the proximal germline nuclei, which normally undergo spermatogenesis in wild-type animals, remain

arrested in the pachytene stage of meiosis I. The more distal nuclei, which presumably undergo oogenesis, also remain arrested in the pachytene phase. Clumped pachytene nuclei can be easily found in the distal arm of the gonad, which might be the result of the diminished size of the gonad.

In addition, adult *sos-1* [RNAi] males generally exhibit short and crumpled spicules (20/20 animals examined) (Figure 3B). *let-60(n1046gf)* but neither a *let-23(sa62gf)* nor a *lin-15(n765ts)* mutation suppresses the lethality and scrawny phenotype conferred by the *sos-1* RNAi, implying that SOS-1 functions downstream of LET-23 and upstream of LET-60. The hyperinduced vulval phenotypes observed in *let-60(n1046gf)*, *let-23(sa62gf)* and *lin-15(n765ts)* mutants are suppressed by the *sos-1* RNAi. However, the suppression of *let-60(n1046gf)* is weaker than that of *let-23(sa62gf)* and *lin-15(n765ts)* based on the distributions of vulval induction (Figure 2; Table I). Since all these phenotypes are also exhibited by mutants in RAS signaling pathway, we conclude that SOS-1 acts on RAS during larval development.

SOS-1 is encoded by let-341

sos-1 maps to the left arm of chromosome V in a region that is close to the estimated position of *let-341* on the genetic map. *let-341* alleles were originally isolated in a screen for essential genes on the left arm of V (Johnsen and Baillie, 1991). In addition, *let-341* alleles were also isolated as extragenic suppressors of the Multivulval phenotype of *lin-15(lf)* mutants (Clark *et al.*, 1992b) and in a screen for suppressors of the Clr (*soc*) phenotype of *clr-1(lf)* mutants (M. Stern, personal communication). Genetic studies suggest that *let-341* has genetic properties consistent with it encoding a RAS exchange factor, namely that it acts downstream of *lin-15* and upstream of *let-60* (Clark *et al.*, 1992b). We therefore

tested whether *let-341* mutants have lesions in *sos-1*. Molecular lesions associated with three *let-341* alleles were found to affect the *sos-1* ORF, indicating that *sos-1* transcript corresponds to *let-341* gene (Figure 1A). *let-341(s1031)* mutants display a 100% embryonic or early larval lethality, which can be suppressed by a *let-60(gf)* mutation. Sequence analysis reveals that *s1031* contains an AG->TT mutation at the splice donor of intron 13 in *sos-1*. The *n2183* mutation, provided by M. Stern, changes a glutamic acid to lysine at codon 331 within the DH domain (Figure 1). The *ku231* allele, provided by Z. Chen and M. Han, results in the substitution of proline for a conserved histidine at codon 881 right before the start of the CDC25 catalytic domain.

s1031 represents a null allele for sos-1

As the molecular lesion associated with *s1031* is predicted to alter splicing, we looked at the *sos-1* mRNA produced in this mutant. We could not detect wild-type *sos-1* mRNA in *s1031* mutants by RT-PCR (Figure 5). The subsequent sequence analysis revealed that in *s1031* animals upstream cryptic splice donors are used and consequently various deletions in the regions encoding the C-terminus of the CDC25 catalytic domain occur (Figure 1C and 5). The truncated transcripts are predicted to encode either a dominant negative or a loss-of-function SOS-1 protein, suggesting that *s1031* completely eliminates SOS-1 activity. Consistent with *s1031* being a null allele, a *sem-5* null or severe reduction-of-function mutation does not further reduce the vulval induction in *let-60(n1046); let-341(s1031)* mutant animals (Table II). To further strengthen this conclusion, we use *sos-1* RNAi to remove any residual *sos-1* activity in *let-60(n1046); let-341(s1031)* mutants. The average vulval induction displayed by the progeny of *sos-1* dsRNA-injected hermaphrodites is 2.6 (n=23), which is not

statistically different from that displayed by animals without *sos-1* dsRNA injection (2.7 (n=54)) (Table I). Together, these three lines of observation provide compelling evidence that *s1031* is a null allele.

Since *s1031* is a null allele, we tested whether this mutation alters vulval induction. We used a *let-60(gf)* mutation to rescue the lethality of *s1031* and found that loss of *let-341 sos-1* function reduces vulval induction. Specifically, 41% of *let-60(n1046); let-341(s1031)* mutant animals exhibit less than wild-type vulval induction, further supporting a role of *sos-1* during vulval induction.

ligand-dependent and SOS-1-independent activation of RAS signaling in the vulva

To test whether *lin-3*-induced vulval signaling is solely mediated by *let-341 sos-1*, we compared the vulval induction in mutant lacking *lin-3* to mutant lacking *let-341 sos-1*. Since LIN-3 produced by the gonadal AC is the only inductive source for vulval differentiation, ablation of gonad eliminates the ligand for *let-23* mediated vulval signaling. The extent of vulval differentiation in *let-60(gf); let-341(null)* mutants is significantly higher than that in gonad-ablated *let-60(gf)* mutants (Table II). This observation suggests that not all of the inductive signaling is mediated through SOS-1. This SOS-1-independent pathway could also be SEM-5 independent since *let-60(gf); sem-5(null)* and *let-60(gf); let-341(null); sem-5(null)* mutants also produce significantly more vulval differentiation than do gonad-ablated *let-60(gf)* mutants. A transgenic line that bears multiple copies of *let-60(n1046gf)* DNA displays completely ligand-independent signaling (Sundaram and Han, 1995). Importantly, this transgenic array does not confer 100% induction in all six VPCs. It has been shown that a weak *lin-45* mutation suppresses the vulval induction conferred by this transgenic line while a weak

sem-5 mutation or the gonad ablation does not. This indicates that gonad ablation does not affect signaling parallel to LET-60 RAS (Sundaram and Han, 1995). Thus, the ligand-dependent and SOS-1-independent pathway most likely acts at the level of LET-60 RAS.

To address the question of whether LET-23 is required for the SOS-1-independent activation of LET-60 RAS, we assayed the extent of vulval induction in *let-23(sy17 null); let-60(n1046)* double mutants. The average vulval induction of *sy17; n1046* strain shows no difference from that of *n1046* single mutants (Table III). Our interpretation of this observation is that there is a LET-23-dependent negative regulation for vulval signaling, which can be lifted by the presence of the gonadal signal. This model is supported by our other experimental results. Because LET-23 has both positive and negative effects on vulval signaling, we can not rule out a LET-23-independent response to the gonadal signal.

To test whether this putative SOS-1-independent pathway is under regulation by SLI-1, we crossed a *sli-1* mutation to a genetic background where the SOS-1-dependent pathway has been removed. SLI-1, similar to mammalian proto-oncoprotein c-Cbl, inhibits LET-23 mediated signaling during vulval development (Jongeward *et al.*, 1995; Yoon *et al.*, 1995). We found that a *sli-1(lf)* mutation fails to enhance the vulval induction in *let-60(gf); let-341(null)* mutants, arguing that SLI-1 does not negatively regulate this other pathway parallel to SOS-1 (Table III). This result also supports our interpretation that *let-341(s1031)* represents a loss-of-function rather than a severe reduction-of-function mutation because we expect that fluctuation in the *ras*-dependent signaling caused by a *sli-1* mutation would result in a dramatic change in vulval induction under the condition where a low level activity of LET-23/SEM-5/SOS-1 is present (C. Yoon, C. Chang and P. Sternberg, unpublished).

Gonad-ablated *let-60(gf); let-341(null)* animals have less vulval differentiation than do *let-60(gf); let-341(null)* or gonad-ablated *let-60(gf)* animals. Therefore, gonad ablation not only removes a ligand-dependent and SOS-1-independent positive signaling pathway but also reveals a ligand-independent activation of RAS by SOS-1. Although this ligand-independent activation of RAS by SOS-1 does not contribute significantly the vulval induction in gonad-ablated *let-60(gf)* animals, it could explain the difference of vulval induction between *let-60(gf); let-341(null)* and *let-60(gf); sem-5(null)* mutants (Table II).

PTP-2 is not involved in SOS-1-independent vulval signaling

Based on the observation that the *loss-of function* mutation in *ptp-2* reduces the vulval induction conferred by a *let-60(gf)* mutation, *ptp-2* was previously interpreted to act in the vulval signaling pathway as a positive effector downstream of, or in parallel to, *let-60 ras* (Gutch *et al.*, 1998). To determine whether the response of PTP-2 to receptor activation is parallel to that of SOS-1, we generated triple mutants with activated RAS but lacking PTP-2 and SOS-1 (*ptp-2(null); let-60(gf); let-341(null)*), and assessed their vulval differentiation. The *ptp-2* mutation did not further reduce the vulval induction observed in *let-60(gf); let-341(null)* mutants, suggesting that PTP-2 does not mediate SOS-1-independent vulval signaling (Table III).

Lifting inhibition by GAP-1 does not explain the SOS-1-independent signaling activity

Besides a SOS-1-independent activation of RAS, an alternative explanation for the elevated signaling activity in the *let-60(gf); let-341(null)* mutant animals is that

activation of LET-23 by the ligand may relieve the inhibition of RAS by GAP-1, GTPase activating protein (Hajnal *et al.*, 1997). In this model, LET-23 would be able to relieve GAP-1-mediated inhibition of RAS upon activation by the ligand in *let-60(gf); let-341(null)* double mutants, while *let-60(gf)* single mutants with an ablated gonad display constitutively active GAP-1 and cause a further decrease in the vulval induction. To test this model, we constructed the triple mutant *let-60(gf); let-341(null); gap-1(null)* and compared the average vulval induction in the conditions with and without the gonad. We find that gonad ablation significantly reduces the vulval induction of *let-60(gf); let-341(null); gap-1(null)* animals (from 3.9 (n=20) to 2.4 (n=13); p=0.0048) (Table II), indicating that the anchor cell signal is not working solely through *let-341* and *gap-1* which are absent already in *let-60(gf); let-341(null); gap-1(null)* animals. This result rules out the possibility that the gonad-dependent *sos-1* independent signaling is mediated solely through *gap-1*.

A role for SOS-1 in FGFR-mediated signaling during development

Hyperactive EGL-15 FGF receptor signaling, conferred by either an activated *egl-15* or a *loss-of-function clr-1* mutation, results in a Clr phenotype. Decreased EGL-15 signaling, conferred by a partial *loss-of-function egl-15* mutation, results in a scrawny body morphology phenotype (Kokel *et al.*, 1998). *clr-1*, which encodes a receptor tyrosine phosphatase, is believed to attenuate signaling from FGFR by dephosphorylation. A scrawny phenotype was observed in the *sos-1* RNAi-affected animals (Figure 4B), suggesting that SOS-1 normally mediates FGFR signaling. To test this possibility, we removed SOS-1 function in the temperature-sensitive *clr-1(e1745ts)* mutants by *sos-1* RNAi and assessed their Clr phenotype. We found that the Clr phenotype of *clr-1(e1745ts)* mutants was suppressed by

sos-1 RNAi (77/77 animals examined) (Figure 4C and D), indicating a positive role for SOS-1 in FGFR-mediated signaling. Indeed, the *let-341(ku231)* mutation also suppresses the Clr phenotype of *clr-1(e1745ts)* mutants (data not shown) and *let-341(n2183)* was isolated as a *soc* mutant, as mentioned before. Since many *sem-5* alleles were also isolated as suppressors of the Clr phenotype of *clr-1(lf)* mutant (Clark *et al.*, 1992a), it is likely that SEM-5-dependent recruitment of SOS-1 to the active FGFR (EGL-15) plays an analogous role in activating RAS as with the EGFR (LET-23).

Discussion

SOS-1 participation during vulval development

We have found the *C. elegans* homolog of Sos, and demonstrated by RNAi and by phenotypic analysis of a null allele, that it is required for vulval induction. *sos-1* RNAi does not affect vulval induction in the wild-type animals at an appreciable frequency (1/50 animals examined vulvaless; data not shown). It might be that vulval induction occurs late in development or a certain property of vulval lineages renders this developmental process more resistant to *sos-1* RNAi. However, the effect of *sos-1* RNAi on vulval signaling becomes obvious in sensitized genetic backgrounds. Although *sos-1* acts downstream of *let-23* and upstream of *let-60*, *sos-1* RNAi affects excessive vulval signaling in both *let-23(gf)* and *let-60(gf)* mutants. The fact that vulval signaling was affected in a greater degree in *let-23(gf)* than *let-60(gf)* mutants is consistent with our expectation that *sos-1* RNAi abrogates both ligand dependent and independent activation by *let-23(gf)* while only abrogates ligand dependent activation by *let-60(gf)*. The involvement of *sos-1* during vulval development is further confirmed by our

observation that 41 percent of the *let-60(gf); let-341(null)* animals examined displayed less than a wild-type extent of vulval differentiation (Table II).

SOS-1 involvement in oocyte maturation and uterine differentiation

Consistent with the observation that *sos-1* RNAi prevents meiotic prophase progression, *sos-1* RNAi-affected hermaphrodites also display a late onset sterile phenotype in the adult stage (10/10 animals examined; data not shown). Furthermore, this sterile phenotype can be suppressed by a *let-60(gf)* mutation (data not shown). Taken together, these results identify SOS-1 as an activator for LET-60 RAS in regulating oogenesis. It remains unknown what is required upstream of SOS-1 to permit exit from pachytene (Church *et al.*, 1995).

As judged by anatomy, we can not tell whether abnormalities in the dorsal and ventral uterine lineages are caused by a mis-determination of cell fates, defects in cell migration, or both. Most of the adult hermaphrodites affected by *sos-1* RNAi have an abnormal everted vulva that consists of vulval cells and somatic gonadal tissues that have been extruded out of the body cavity (data not shown). Uterine cells are known to be required for the proper attachment between the uterus and vulva (Newman and Sternberg, 1996), a process that has been suggested to prevent the vulva and uterus from slipping out of the body cavity during vulval eversion (Seydoux *et al.*, 1993).

SOS-1-dependent and independent signaling

The finding that the *sli-1(null)* mutation fails to enhance the vulval induction in *let-60(gf); let-341(null)* mutants suggests that the synergy between *sli-1(null)* and *let-60(gf)* in the vulva is dependent upon SOS-1. This result is consistent with our

previous data suggesting that *sli-1* acts upstream of *let-60 ras* to inhibit signaling from *let-23* (C. Yoon, C. Chang and P. Sternberg, unpublished). The finding that the extent of vulval induction in a *let-60(gf); let-341(null)* background is higher than in gonad ablated *let-60(gf)* animals reveals that there is a ligand-dependent and SOS-1-independent signaling by EGF RTK. The simplest explanation is that there is a SOS-1-independent positive signaling pathway in *let-60(gf); let-341(null)* animals. Alternatively, gonad-ablated *let-60(gf)* animals may be subject to a form of negative regulation in the vulva that is independent of SOS-1 and is lifted in a *let-60(gf); let-341(null)* background perhaps by the presence of the ligand. A SOS-1-independent positive signaling pathway elicited by LIN-3 might involve another guanine nucleotide exchange factor for RAS or the DOS/PTP-2-cascade. It has been previously shown that there may be more than one activator of LET-60 RAS. Genetic analysis of *sur-5* revealed that a *sur-5(lf)* mutation selectively suppresses one group of *let-60(dn)* alleles but not the other, supporting the hypothesis that there could be more than one activator for LET-60 RAS (Gu *et al.*, 1998b). *let-60(dn)* alleles have been shown to affect signaling by titrating upstream activators rather than downstream effectors (Han and Sternberg, 1991). At least seven additional putative guanine nucleotide exchange factors have been predicted from the *C. elegans* genome sequence (as of Apr. 2000), and one or more of these could be the hypothetical activator.

Regulation of RTK pathways by PTP-2

Csw and SHP-2 are PTP-2 homologs in *Drosophila* and vertebrates, respectively. Csw is normally required for Sevenless, Breathless, Torso, and DER pathways while SHP-2 has been shown to function in EGFR, PDGFR, FGFR, and HGFR signaling pathways. The best characterized substrate for Csw/SHP-2 are

Daughter of sevenless (Dos)-type proteins (Gu *et al.*, 1998a; Herbst *et al.*, 1996; Raabe *et al.*, 1996). It is believed that dephosphorylation of Dos by Csw generates a positive downstream signal. However, it remains unclear how Dos dephosphorylation creates such a positive signal. It is conceivable that PTP-2 might dephosphorylate specific phospho-tyrosyl residue(s) on a Dos-like protein, thus controlling the composition of an assembled signaling complex. Based on our results that PTP-2 function could be dependent upon SOS-1, several models for PTP-2 action can be proposed (Figure 6). PTP-2 could modulate the SEM-5-directed recruitment of SOS-1 or the catalytic activity of SOS-1. Alternatively, PTP-2 could release a negative regulator, such as SLI-1, from the signaling complex.

SOS-1 acts in multiple, RAS-dependent signaling pathways

SEM-5 recruitment of SOS-1 to the cell membrane seems to be a common mechanism involved in transducing signals from the activated EGFR and FGFR to RAS (Figure 6). Although SOS-1 also participates in signaling during the maturation of germline cells, how it is directed and to what signal it responds are unclear. Regulation of signaling upstream of RAS has been intensively studied from EGFR by using vulval development as a major paradigm. Several negative regulators have been previously implicated as affecting RAS activation by LET-23, including SLI-1/Cbl, UNC-101/AP47, and GAP-1/RasGAP. Biochemical studies suggest that RasGAP inhibits the signaling by directly acting on Ras (Boguski and McCormick, 1993), while Cbl desensitizes the signaling through binding to active receptors (Meisner *et al.*, 1997). It is not clear how the UNC-101 adaptor antagonizes the RAS signaling pathway. In this report, we have provided evidence for the existence of a SOS-1-independent signaling pathway,

which is dependent upon ligand, independent of PTP-2, and is not negatively regulated by SLI-1. Recently, Cbl was shown to function as a ubiquitin-protein ligase for active RTKs (Joazeiro *et al.*, 1999). The failure to see an effect of SLI-1 on the SOS-1-independent pathway argues that promoting the degradation of active EGFR might not be the major activity of SLI-1. SLI-1 and another negative regulator of LET-23 EGFR signaling, ARK-1, both have proline-rich domains that can interact with the SH3 domain of SEM-5 (C. Yoon, C. Chang, P. Sternberg, unpublished; N. Hopper, J. Lee and P. Sternberg, in preparation). Competition between SOS-1 and negative regulators for binding to the SEM-5 adaptor is a potential mechanism of direct negative regulation of LET-60 RAS activation. Alternatively, the SH3 domains of SEM-5 may, in addition to the recruitment of SOS-1, also recruit negative regulators simultaneously with SOS-1. It is less likely that *ptp-2* activates signaling by inhibiting *gap-1* function, since a *ptp-2* mutation did not further reduce the vulval induction in *let-60(gf); let-341(null)* mutants, and a *let-60(gf); let-341(null)* mutant is sensitive to *gap-1* regulation (Table II). Regulation of Sos1 by phosphorylation has been proposed previously (Buday *et al.*, 1995; Hu and Bowtell, 1996). Enhanced phosphorylation of Sos1, while not altering its catalytic activity for Ras, weakens its binding affinity for Grb2 or uncouples the Sos1/Grb2 complex from tyrosine kinase substrates (Buday and Downward, 1993). Although Sos1 is phosphorylated mostly on serine and threonine, regulated tyrosine phosphorylation has never been ruled out for Sos1. Therefore, it is possible that protein tyrosine kinases/phosphatases might modulate RAS signaling strength by directly regulating the phosphorylation state of SOS-1.

Materials and methods

Strains

Strains were handled according to the standard protocol and maintained at 20°C (Brenner, 1974). The following alleles were used: for LGII, *let-23(sa62)*, *ptp-2(op194)* (Gutch *et al.*, 1998), *unc-4(e120)*, *clr-1(e1745)* (Hedgecock *et al.*, 1990); for LGIV, *let-60(n1046)* (Ferguson and Horvitz, 1985), *unc-31(e169)*; for LGV, *him-5(e1490)*, *let-341(s1031)* (Johnsen and Baillie, 1991), *let-341(n2183)* (M. Stern, unpublished), *let-341(ku231)* (Z. Chen and M. Han, unpublished); for LGX, *lin-15(n765)*, *sem-5(ay73)* (M. Stern, unpublished), *gap-1(n1691)* (Hajnal *et al.*, 1997), *sli-1(sy143)*.

Construction of double and triple mutants

Double and triple mutants were constructed using standard genetic methods, and markers used are indicated in the tables. For *let-60(n1046); let-341(s1031)*, *let-60(n1046); sli-1(sy143)* double mutants and for *let-60(n1046); let-341(s1031); sem-5(ay73)*, *let-60(n1046); let-341(s1031); gap-1(n1691)*, *let-60(n1046); let-341(s1031); sli-1(sy143)*, *ptp-2(op194); let-60(n1046); let-341(s1031)* triple mutants, the presence of *let-341*, *sli-1*, *sem-5*, and *gap-1* mutations was confirmed by sequencing the appropriate region of genomic DNA from each strain.

sos-1 cDNA isolation and allele sequencing

Partial *sos-1* cDNA was derived from an incomplete genomic sequence, which was provided by *C. elegans* sequencing consortium (1998) and predicted to encode a Sos homolog using the program GeneFinder. RT-PCR reactions were performed to rectify the GeneFinder prediction and confirmed our further

derivation based on the comparative analyses. Two splice variants were recovered in addition to the major transcript. One variant results from failure to use the normal splice donor site of intron 12. The other results from usage of an alternative splice acceptor site in intron 13 (AG/GAATTGCCGAACATATACTAATCACTCTAA). 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions were carried out as manufacturer's instructions to obtain sequences corresponding to the 5′ and 3′ ends of the *sos-1* transcript (5′/3′ RACE kit, BOEHRINGER MANNHEIM).

To identify molecular lesions, PCR was performed on both reverse-transcribed RNA and genomic DNA from mutants, the products were purified and sequenced. The consequence of splice site mutation was determined by PCR amplification of reverse-transcribed RNA from mutants and sequencing purified PCR fragments directly.

RNAi

dsRNA corresponding to *sos-1* was generated by *in vitro* transcription using 1.6-kb *sos-1* cDNA corresponding to the nucleotides 1,045 to 2,613 relative to the initiation codon inserted into pCRII-TOPO (Invitrogen). Transcripts were prepared using T7 and Sp6 RNA polymerase and annealed prior to injection (Fire *et al.*, 1998). Progeny of injected animals were assayed at 20°C unless indicated otherwise.

Vulval induction assay

The extent of vulval induction was determined by examining vulval anatomy in the early to mid-L4 stage of development under Nomarski optics using a Plan

100 objective (Sternberg and Horvitz, 1986). Wild-type animals have three VPCs generating vulval progeny; vulvaless animals have fewer than three VPCs generating vulval progeny; multivulva or hyperinduced animals have more than three VPCs generating vulval progeny. In some cases, a VPC generates one daughter that makes vulval progeny and another daughter that becomes nonvulval epidermis; such VPCs are scored as one-half VPC differentiating into vulval tissue.

Cell ablation

Ablations of the AC was performed with a laser microbeam as described by Sulston and White (1980).

DAPI staining

Animals were washed in M9 buffer before picked into a small drop of Vectashield with 4′,6-diamidino-2-phenylindole (Vector laboratories) in a depression slide. After 30-60 minutes at room temperature, animals were dried in a vacuum for one hour before mounted on agarose pads for observation.

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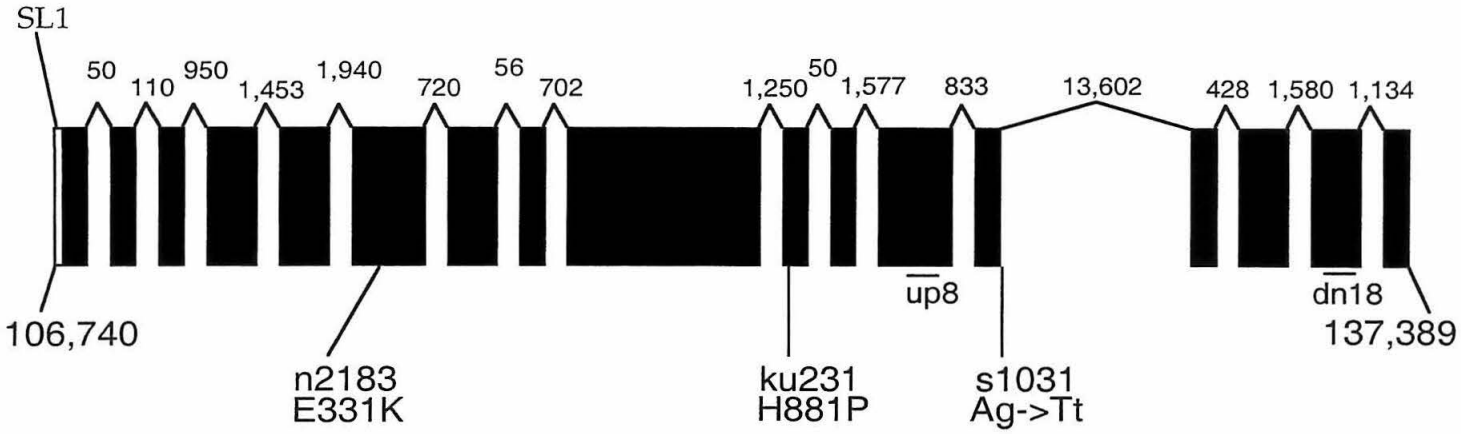
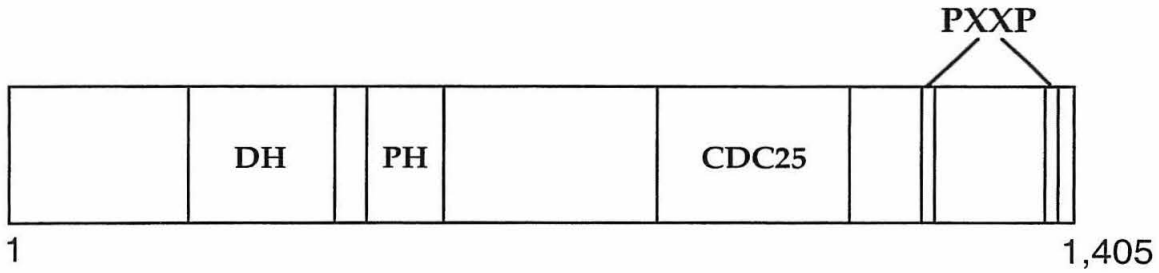
Fig. 1.

(*A*) Genomic structure of the *sos-1* locus. The solid bars represent exons, which were inferred by comparing the nucleotide sequence of a *sos-1* cDNA with that of the genomic DNA; 5′ untranslated region is depicted as an open box. Sizes of the 16 introns are noted by the numbers above each intron. The start and tentative end of the *let-341 sos-1* coding sequences are indicated by the corresponding nucleotide numbers from YAC Y61A9LA. Diagonal line labeled SL1 trans-spliced leader attachment site. The positions of *let-341* mutations and corresponding amino acid or splice site changes are shown. Exonic sequence is shown in uppercase lettering, intronic sequence in lowercase. The approximate locations of primers up8 and dn18 used to analyze *s1031* transcripts are indicated. (*B*) Comparative analyses of signature motifs in SOS-1 homologs. The protein predicted to be encoded by the *sos-1* gene is depicted schematically. The numbers listed below represent the percent identity between DH domain, PH domain, and CDC25 catalytic domain of SOS-1 and those domains of human Sos1 and *Drosophila* sos. Motifs were identified using the ProfileScan program. (*C*) Sequence alignment of CDC25 domains of RAS-binding exchange factors from *C. elegans* (Ce), *Drosophila melanogaster* (Dm), and Homo sapiens (Hs) with residue numbers of *C. elegans* (Ce) SOS-1 indicated. Residue identity between species is highlighted in black, and similarity is highlighted in gray. The arrows indicate the start of deletions in the *s1031* transcripts.

A

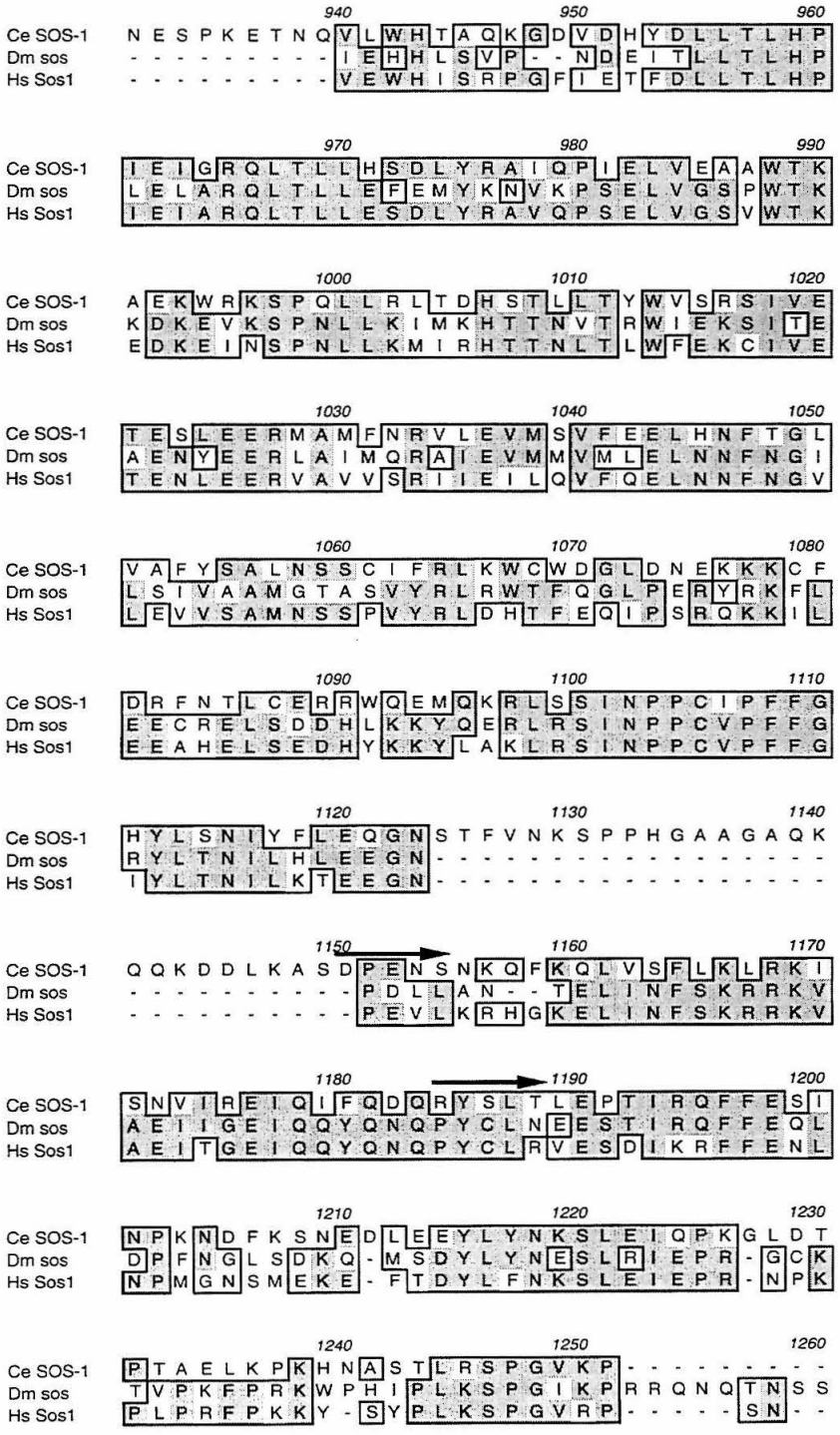


B



human Sos1	26	23	43
***Drosophila* sos**	23	25	39

C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Hs Sos1 - - - - - - - - - - V E W H I S R P G F I E T F D L L T L H P
970 980 990
Ce SOS-1 I E I G R Q L T L L H S D L Y R A I Q P I E L V E A A W T K
Dm sos L E L A R Q L T L L E F E M Y K N V K P S E L V G S P W T K
Hs Sos1 I E I A R Q L T L L E S D L Y R A V Q P S E L V G S V W T K
1000 1010 1020
Ce SOS-1 A E K W R K S P Q L L R L T D H S T L L T Y W V S R S I V E
Dm sos K D K E V K S P N L L K I M K H T T N V T R W I E K S I T E
Hs Sos1 E D K E I N S P N L L K M I R H T T N L T L W F E K C I V E
1030 1040 1050
Ce SOS-1 T E S L E E R M A M F N R V L E V M S V F E E L H N F T G L
Dm sos A E N Y E E R L A I M Q R A I E V M M V M L E L N N F N G I
Hs Sos1 T E N L E E R V A V V S R I I E I L Q V F Q E L N N F N G V
1060 1070 1080
Ce SOS-1 V A F Y S A L N S S C I F R L K W C W D G L D N E K K K C F
Dm sos L S I V A A M G T A S V Y R L R W T F Q G L P E R Y R K F L
Hs Sos1 L E V V S A M N S S P V Y R L D H T F E Q I P S R Q K K I L
1090 1100 1110
Ce SOS-1 D R F N T L C E R R W Q E M Q K R L S S I N P P C I P F F G
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1120 1130 1140
Ce SOS-1 H Y L S N I Y F L E Q G N S T F V N K S P P H G A A G A Q K
Dm sos R Y L T N I L H L E E G N - - - - - - - - - - - - - - - - - -
Hs Sos1 I Y L T N I L K T E E G N - - - - - - - - - - - - - - - - - -
1150 1160 1170
Ce SOS-1 Q Q K D D L K A S D P E N S N K Q F K Q L V S F L K L R K I
Dm sos - - - - - - - - - - P D L L A N - - T E L I N F S K R R K V
Hs Sos1 - - - - - - - - - - P E V L K R H G K E L I N F S K R R K V
1180 1190 1200
Ce SOS-1 S N V I R E I Q I F Q D Q R Y S L T L E P T I R Q F F E S I
Dm sos A E I I G E I Q Q Y Q N Q P Y C L N E E S T I R Q F F E Q L
Hs Sos1 A E I T G E I Q Q Y Q N Q P Y C L R V E S D I K R F F E N L
1210 1220 1230
Ce SOS-1 N P K N D F K S N E D L E E Y L Y N K S L E I Q P K G L D T
Dm sos D P F N G L S D K Q - M S D Y L Y N E S L R I E P R - G C K
Hs Sos1 N P M G N S M E K E - F T D Y L F N K S L E I E P R - N P K
1240 1250 1260
Ce SOS-1 P T A E L K P K H N A S T L R S P G V K P - - - - - - - - -
Dm sos T V P K F P R K W P H I P L K S P G I K P R R Q N Q T N S S
Hs Sos1 P L P R F P K K Y - S Y P L K S P G V R P - - - - - S N - -

Fig. 2.

Suppression of excessive vulval signaling by *sos-1* RNAi. Nomarski photomicrographs of the vulval induction in the L4 stage hermaphrodites. (*A*) *lin-15(n765)* mutant, which displays a total of six VPCs adopting vulval fates and forms three vulval invaginations. (*B*) *sos-1* RNAi-affected *lin-15(n765)* mutant, which displays a one cell induction that forms one vulval invagination. (*C*) *let-23(sa62)* mutant, which displays a total of four VPCs undergoing vulval differentiation and forms two vulval invaginations. (*D*) *sos-1* RNAi-affected *let-23(sa62)* mutant, which displays only two induced vulval cells that form one vulval invagination. (*E*) *let-60(n1046)* mutant, which displays a total of five VPCs adopting vulval fates and forms three vulval invaginations. (*F*) *sos-1* RNAi-affected *let-60(n1046)* mutant, which displays a wild-type vulval induction and invagination. Dorsal is up and anterior to the right in all panels. The scale bar is 20 μm.

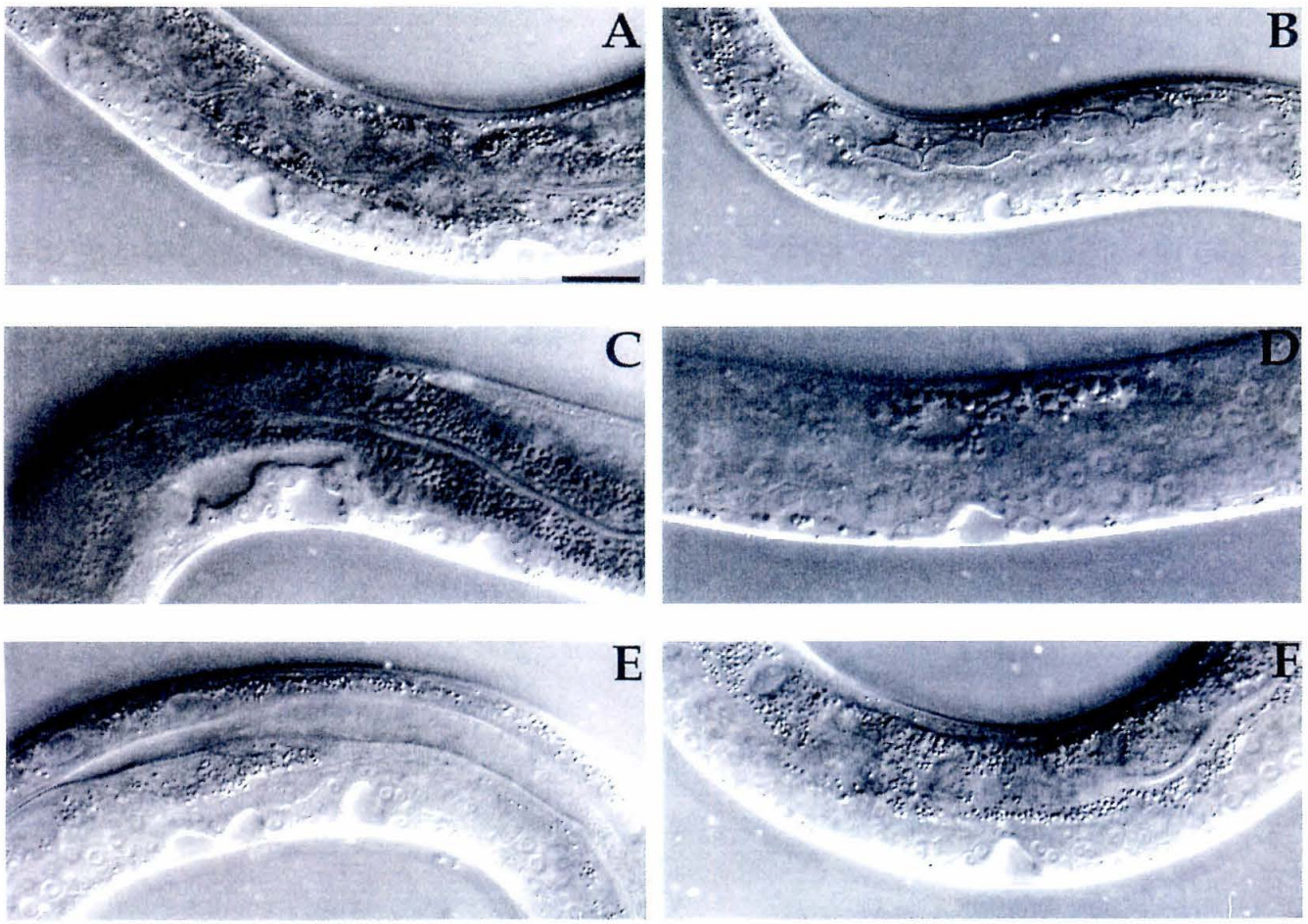

A
B
C
D
E
F

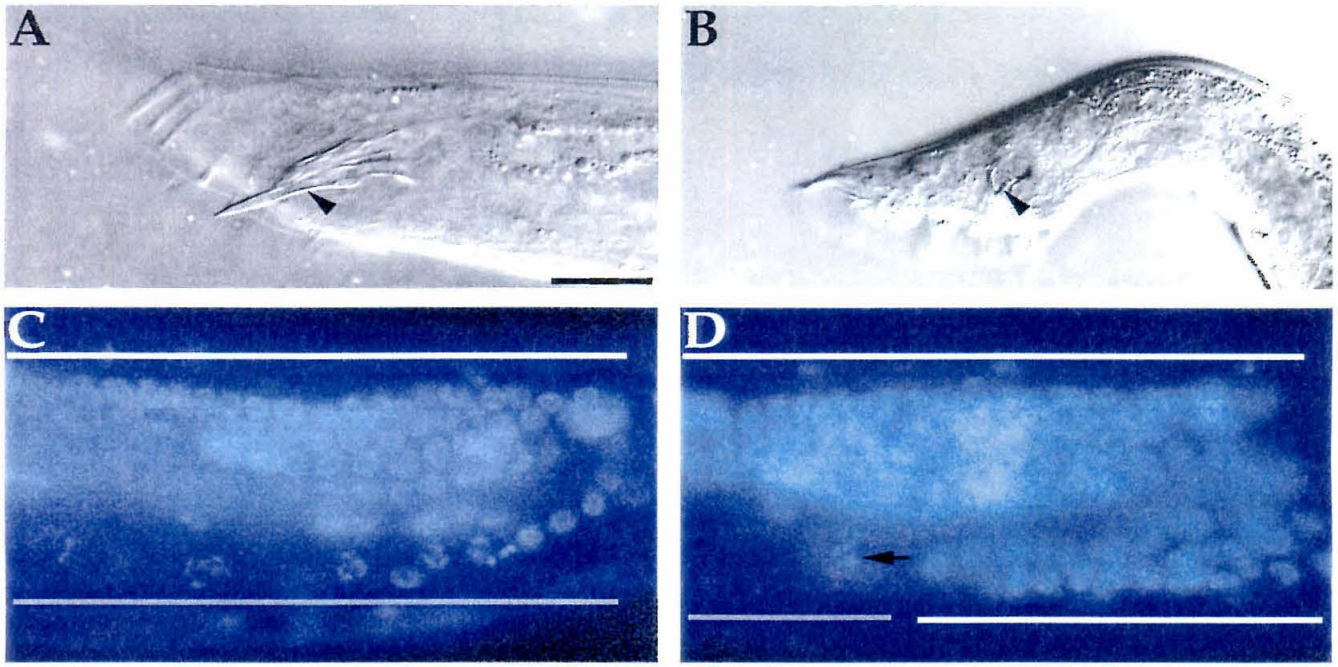
Table I. *sos-1* mediates the ectopic vulval induction during excessive *let-23* signaling

Genotype	*sos-1* RNAi	%<3	%=3	%>3	Numbers of animals	Average vulval induction
let-60(n1046)	-	0	0	100	20	5.0
let-60(n1046)	RNAi	0	62	38	21	3.3
let-23(sa62)	-	0	7	93	15	4.2
let-23(sa62)	RNAi	14	72	14	14	2.9
lin-15(n765)	-	0	0	100	20	5.3
lin-15(n765)	RNAi	33	67	0	12	2.5
let-60(n1046); let-341(s1031)	-	41	52	7	54	2.7
let-60(n1046); let-341(s1031)	RNAi	39	52	9	23	2.6

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. The complete genotypes for strains listed are: *let-60(n1046)unc-31(e169)* [for *let-60(n1046)*]; *let-23(sa62); him-5(e1490)* [for *let-23(sa62)*]; *lin-15(n765)* [for *lin-15(n765)*]; *let-60(n1046); let-341(s1031)unc46(e177)* [for *let-60(n1046); let-341(s1031)*]. Average vulval induction represents average number of VPCs undergoing vulval differentiation per animal; 3.0 is wild-type level.

Fig. 3.

sos-1 RNAi affects male spicule development and hermaphrodite oocyte maturation. (*A*) Nomarski photomicrograph of a *him-5(e1490)* male shows a long and straight spicule, denoting by an arrow head. (*B*) A male progeny of *him-5(e1490)* hermaphrodite injected with *sos-1* dsRNA shows a short and crumpled spicule, denoting by an arrow head. (*C,D*) Fluorescence micrograph of wild-type (bottom left) and *sos-1* RNAi-affected (bottom right) hermaphrodites stained with DAPI. Regions of pachytene nuclei are indicated by a white bar and nuclei in diakinesis by a semi-gray bar. Arrow indicates the first diakinesis nucleus seen in the *sos-1* RNAi-affected hermaphrodite. All animals shown are in their young adult stage. Dorsal is up and anterior to the right in all panels. The scale bar is 20 μm.


A
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D

Fig. 4.

sos-1 RNAi-affected animals display the phenotypes associated with *reduction-of-function* FGFR pathway mutants. All the animals were photographed at the same magnification. (*A*) Wild type (N2). (*B*) *sos-1* RNAi-affected N2 animal, which confers a scrawny body morphology. (C) *clr-1(e1745ts)* mutants, which confer a temperature-sensitive Clr phenotype. The biological basis of the Clr phenotype is not understood but the abnormality appears to result from clear fluid accumulating in the pseudocoelom (Kokel *et al.*, 1998). (*D*) *sos-1* RNAi-affected *clr-1(e1745ts)* mutant, which confers a non-Clr phenotype. Animals shown in *A* and *B* are young adults and were raised at 20°C. L4 larvae shown in *C* and *D* were raised at 20°C and then switched to the nonpermissive temperature of 25°C for 10 hour before being photographed.

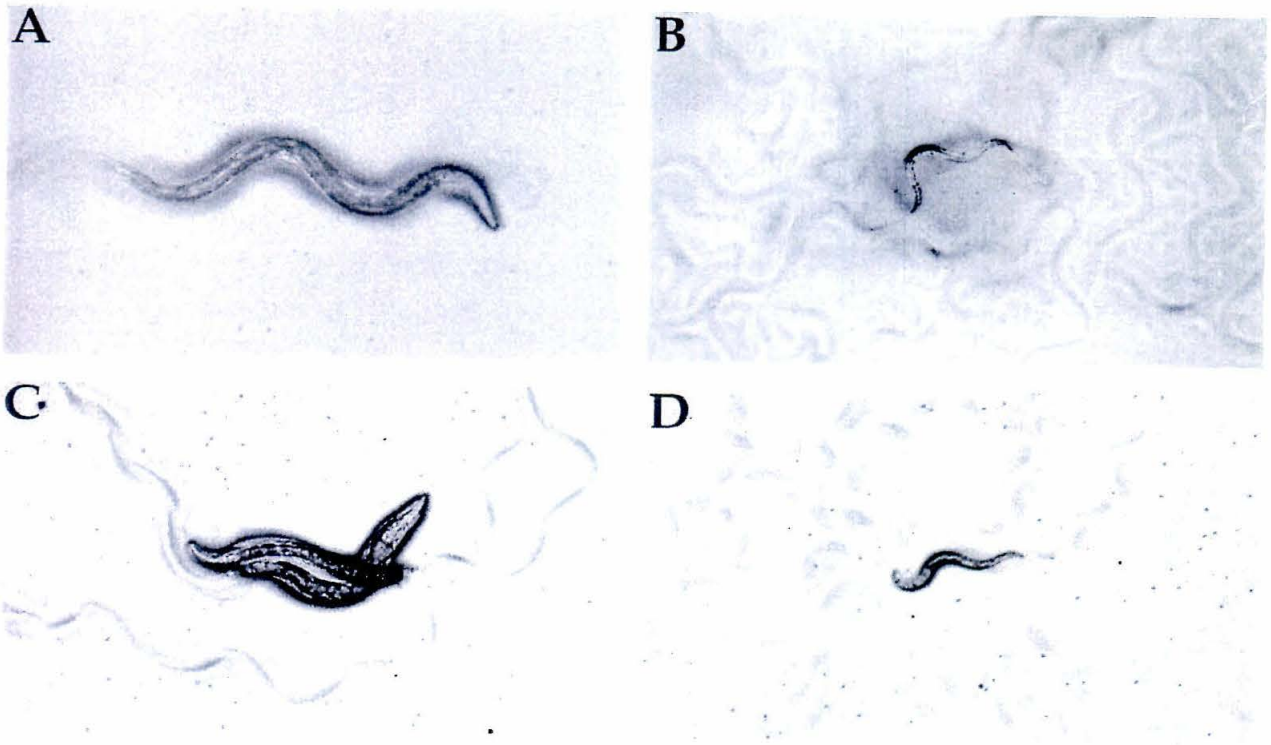
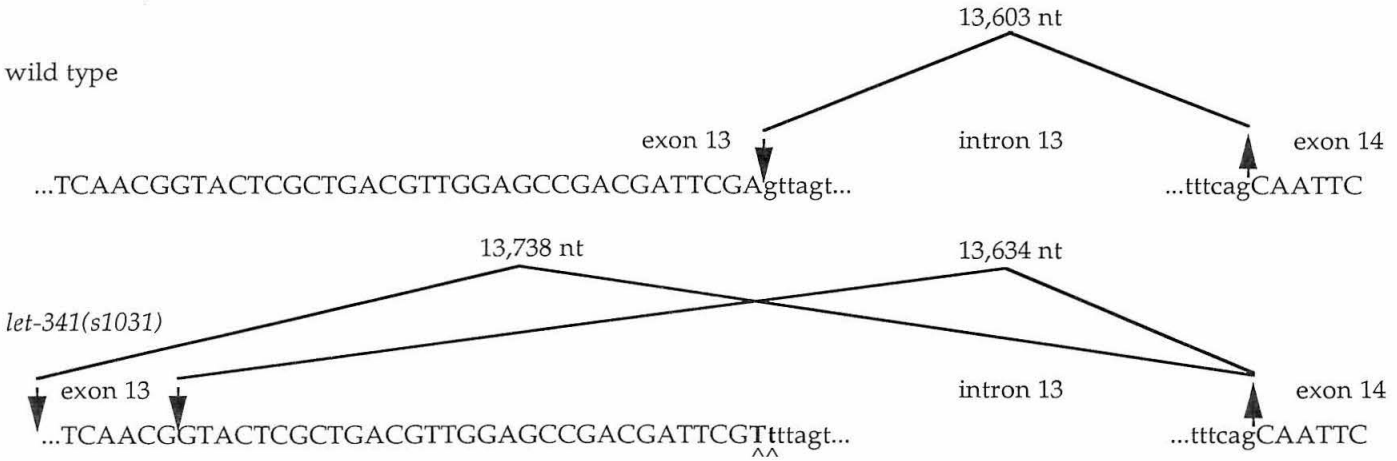

A
B
C
D

Fig. 5.

let-341(s1031) is a putative null allele of *sos-1*. (*A*) The sequence of the *let-341(s1031)* mutation. The upper panel shows the genomic sequence around the boundaries of exon 13-intron 13 and intron 13-exon 14. Exons are shown in uppercase lettering, introns in lowercase. The large downward-pointing arrow indicates the 5′ location of the wild-type splice. The upward-pointing arrow indicates the 3′ splice junction used. The lower panel shows the same information in the presence of the *s1031* mutation. The mutation is indicated in bold and by carets. The 5′ splice donors have shifted upstream. (*B*) RT-PCR analysis of wild-type and *s1031* RNA. PCR amplification for 35 rounds of wild-type and *s1031* reverse-transcribed RNA was performed with primers up8 and dn18 (see Figure 1A). Lanes: 1, 1-kb ladder; 2, amplification of *s1031* cDNA; 3, amplification of wild-type cDNA. Correctly spliced mRNA yields a product of 678 bp (correct joining of exons 12-16). In *s1031* animals, a 647 bp product results from usage of a upstream cryptic splice donor at nucleotide 119,792 of YAC Y61A9LA and removal of 31 bp from exon 13. Exon 13 skipping in *s1031* animals results in a 543 bp product. A putative heteroduplex (het), expected to form from two fast migrating species, is indicated.

A


13,603 nt
wild type
exon 13
intron 13
exon 14
...TCAACGGTACTCGCTGACGTTGGAGCCGACGATTCGAgttagt...
...tttcagCAATTC
13,738 nt
13,634 nt
let-341(s1031)
exon 13
intron 13
exon 14
...TCAACGGTACTCGCTGACGTTGGAGCCGACGATTCGTttagt...
...tttcagCAATTC

B

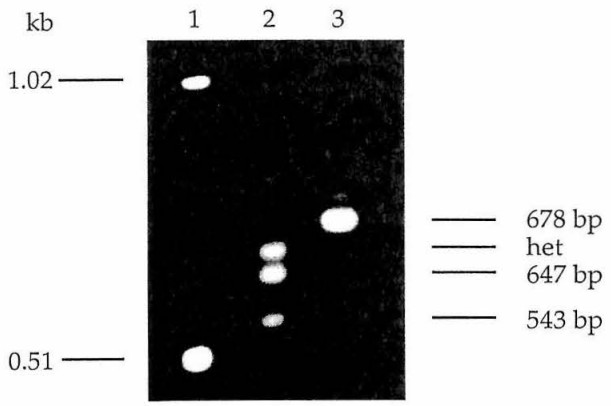

kb
1
2
3
1.02
0.51
678 bp
het
647 bp
543 bp

Table II. A *sos-1*-independent activation of *ras* signaling in the vulva

Genotype					%<3	%=3	%>3	Numbers of	Average Vulval
let-60	*let-341*	*gap-1*	*sem-5*	*gonad*				animals	induction
1. *n1046*	+	+	+	+	0	15	85	20	4.4
2. *n1046*	+	+	+	-	89	0	11	18	1.3
3. *n1046*	*s1031*	+	+	+	41	52	7	54	2.7
4. *n1046*	+	+	*ay73*	+	0	87	13	30	3.2
5. *n1046*	*s1031*	+	*ay73*	+	50	25	25	8	2.8
6. *n1046*	*s1031*	+	+	-	95	5	0	22	0.8
7. *n1046*	*s1031*	*n1691*	+	+	0	35	65	20	3.9
8. *n1046*	*s1031*	*n1691*	+	-	54	15	31	13	2.4

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. *let-341(s1031)* was marked with *unc-46(e177)*. *sem-5(ay73)* is a putative null allele resulting in a Q10Stop (Clark *et al.*, 1992a; M. Stern, personal communication). *gap-1(n1691)* corresponds to a Q149Stop which defines a putative null allele. The distributions generated from each genotype were entered into the InStat program (GraphPad Software, version 2.0) and compared in pairs using the Mann-Whitney test to generate two-tailed P values. The differences result from the following comparisons are considered very significant. 2 versus 3, P value < 0.0001; 2 versus 4, P value < 0.0001; 2 versus 5, P = 0.0021; 3 versus 6, P value < 0.0001; 3 versus 7, P value < 0.0001; 7 versus 8, P = 0.0048. With a P value of 0.7448, 3 versus 5 is considered not significant.

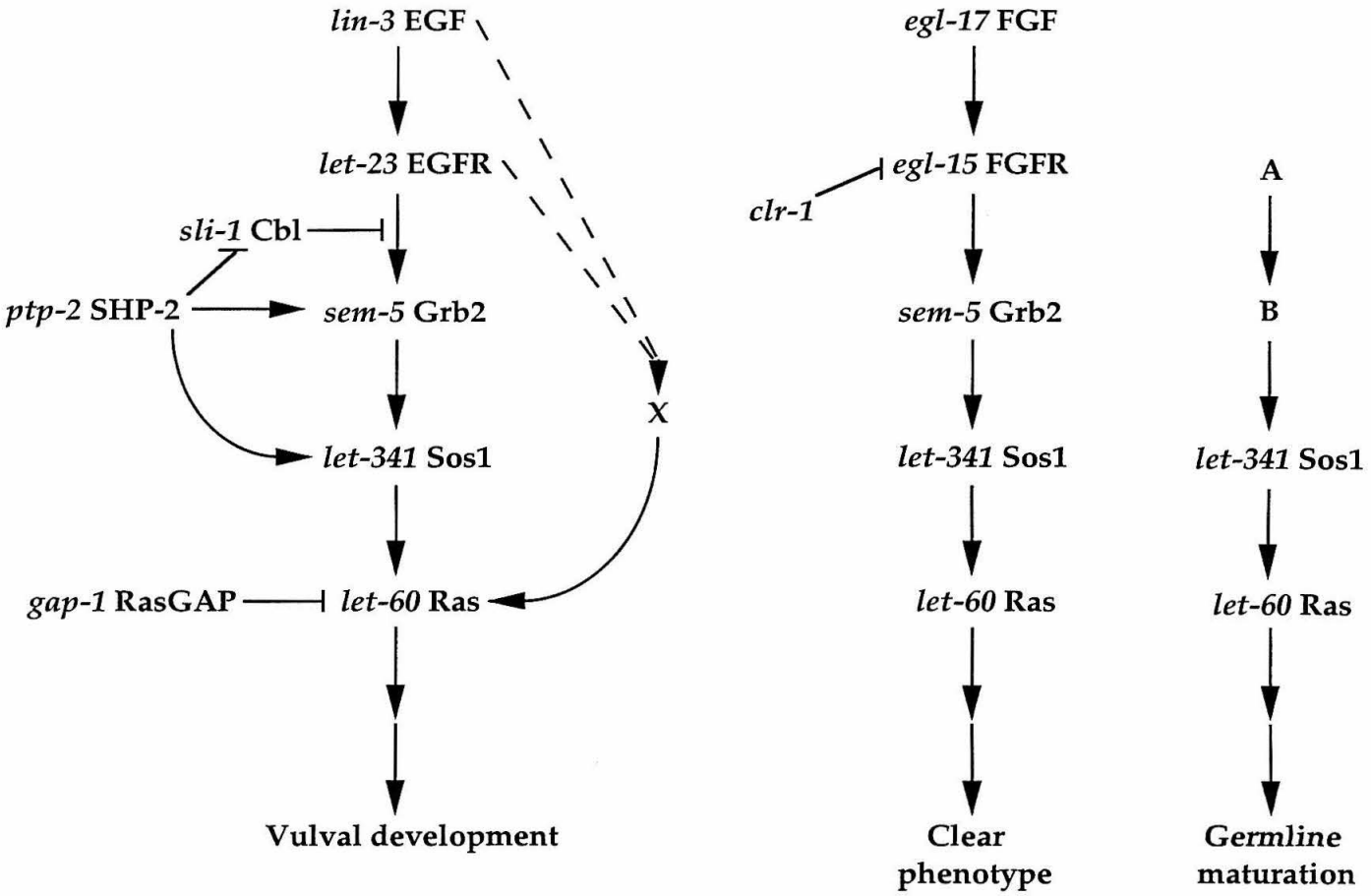
Table III. *sli-1* regulates *let-23* signaling upstream of *ras*, in a *sos-1*-dependent manner

	Genotype				Average vulval	Proportion of animals
ptp-2	*let-23*	*let-60*	*let-341*	*sli-1*	induction	with >3 cells induced
1. +	+	*n1046*	+	+	4.4*	17/20
2. +	*sy17*	*n1046*	+	+	4.6	19/20
3. +	+	*n1046*	+	*sy143*	5.5*	20/20
4. +	+	*n1046*	*s1031*	+	2.7	4/54
5. +	+	*n1046*	*s1031*	*sy143*	2.9	1/20
6. *op194*	+	*n1046*	*s1031*	+	3.0	2/20

Vulval induction was scored at 20°C using Nomarski optics during the early-mid L4 stage. *ptp-2(op194)* and *let-341(s1031)* were marked with *unc-4(e120)* and *unc-46(e177)*, respectively. *ptp-2(op194)* is a putative null allele resulting in a genomic deletion of 1458 nucleotides (Gutch *et al.*, 1998). *sli-1(sy143)* is also a putative null allele resulting in a Q152Stop (Yoon *et al.*, 1995). *The difference of average vulval induction between *let-60(n1046)* and *let-60(n1046); sli-1(sy143)* mutants is highly significant (P = 0.0002) based on the Mann-Whitney test. The most significant difference occurs in the P8.p induction with 33% of P8.p induced for *let-60(n1046)* and 78% *for let-60(n1046); sli-1(sy143)*. The differences result from the following comparisons are considered not significant. 4 versus 5, P value is 0.1629; 4 versus 6, P = 0.1313.

Fig. 6.

Models for EGFR, FGFR, and an unknown RTK pathways. The order of linear pathways was either determined by double mutants analyses or inferred from biochemical assays in other model systems. For example, the relationship between *sem-5* Grb2 and *let-341* Sos1 was adapted from the previous molecular characterization of mammalian counterparts. Arrows indicate a positive effect and bars indicate a negative effect. For simplicity, only events upstream of RAS are presented. The dashed pathway X is insufficient to allow signaling in the absence of *let-60* activity. The putative relationships between *ptp-2* and target genes are yet to be defined. A, B and X are hypothetical receptor tyrosine kinase, adaptor and RasGEF, respectively.


lin-3 EGF
let-23 EGFR
sli-1 Cbl
ptp-2 SHP-2
sem-5 Grb2
X
let-341 Sos1
gap-1 RasGAP
let-60 Ras
Vulval development
egl-17 FGF
egl-15 FGFR
clr-1
sem-5 Grb2
let-341 Sos1
let-60 Ras
Clear phenotype
A
B
let-341 Sos1
let-60 Ras
Germline maturation

Chapter 3

Requirements of multiple domains of SLI-1, a *C. elegans* homolog of c-Cbl, and an inhibitory tyrosine in LET-23 in regulating vulval differentiation

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SLI-1, a *C. elegans* homolog of the proto-oncogene product c-Cbl, is a negative regulator of LET-23-mediated vulval differentiation. Lack of SLI-1 activity can compensate for decreased function of the LET-23 EGFR, the SEM-5 adaptor but not the LET-60 RAS, suggesting that SLI-1 acts prior to RAS activation. SLI-1 and c-Cbl comprise an N-terminal region (termed SLI-1:N/Cbl-N containing a four helix bundle, an EF hand and a divergent SH2 domain) followed by a RING finger domain and a proline rich C-terminus. In a transgenic functional assay the proline rich C-terminal domain is not essential for *sli-1*(+) function. A protein lacking the SH2 and RING finger domains has no activity, but a chimeric protein with the SH2 and RING finger domains of SLI-1 replaced by the equivalent domains of c-Cbl has activity. The RING finger domain of c-Cbl has been recently shown to enhance ubiquitination of active RTKs by acting as an E3 ubiquitin-protein ligase. We find that the RING finger domain of SLI-1 is partially dispensable. Further, we identify an inhibitory tyrosine of LET-23 requiring *sli-1*(+) for its effects: removal of this tyrosine closely mimics loss of *sli-1* but not of another negative regulator, *ark-1*. Thus we suggest that this inhibitory tyrosine mediates its effects through SLI-1, which in turn inhibits signaling upstream of LET-60 RAS in a manner not wholly dependent upon the ubiquitin-ligase domain.

INTRODUCTION

Receptor tyrosine kinase (RTK)/Ras/MAPK signaling pathways are functionally conserved among metazoans in various aspects of cell growth and differentiation (Fantl et al., 1993; Schlessinger and Ullrich, 1992). As unregulated RTK signaling can promote abnormal cell growth or differentiation, precise control of both the duration and the level of RTK activation is important (Cantley et al., 1991; Rodrigues and Park, 1994) and thus it is crucial to understand the negative regulation of RTK signaling.

RTK-mediated signal transduction is initiated by ligand binding, dimerization and subsequent autophosphorylation of cytoplasmic tyrosines within the C-terminal of the receptor (Claesson-Welsh, 1994; Fantl et al., 1993; Ullrich and Schlessinger, 1990). Phosphotyrosine (pTyr) sites in activated RTKs recruit the Grb2/SEM-5/Drk adaptor protein (Clark et al., 1992; Lowenstein et al., 1992; Oliver et al., 1993; Simon et al., 1993). This adaptor protein is associated with the Sos guanine nucleotide exchange factor (GNEF) (Egan et al., 1993; Gale et al., 1993; Li et al., 1993). Once recruited to the membrane, Sos activates the membrane-bound Ras protein by enhancing the exchange of GTP for GDP (Aronheim et al., 1994; Gale et al., 1993; Li et al., 1993; Quilliam et al., 1994; Rozakis-adcock et al., 1993; Simon et al., 1991). Activated Ras recruits the Raf serine/threonine kinase to the membrane where Raf is activated by unknown mechanisms (Campbell et al., 1998; Rommel and Hafen, 1998). Raf phosphorylates and activates MAPK kinase (MEK), which in turn phosphorylates and activates MAPK (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992; Tsuda et al., 1993), leading to diverse cellular responses. The development of the *Caenorhabditis elegans* vulva provides a readily accessible genetic system for the study of RTK signaling and regulation *in vivo* (Chang and Sternberg, 1999; Kornfeld, 1997; Sternberg and Han, 1998; Sundaram and Han, 1996). The wild-type vulva is derived from precisely three of six multipotential vulval precursor cells (VPCs) that generate vulval tissue when induced by the gonadal

anchor cell (Horvitz and Sternberg, 1991). The induced VPCs undergo three rounds of division and a characteristic morphogenesis to form the vulva. VPCs that do not receive adequate signal from the anchor cell divide only once and become part of the hyp7 syncytial epidermis. The LIN-3 protein has a single epidermal growth factor (EGF) domain and is produced by the anchor cell (Hill and Sternberg, 1992). LET-23, a homolog of the EGF receptor (EGFR) and likely receptor for LIN-3, is necessary for not only vulval differentiation but also other aspects of development (Aroian et al., 1990; Aroian and Sternberg, 1991; Chang et al., 1999; Clandinin et al., 1998; Jiang and Sternberg, 1998). LET-23 activation initiates a signaling cascade that involves SEM-5 (Grb2), LET-341 SOS-1 (Sos), LET-60 (Ras), LIN-45 (Raf), MEK-2 (MAPK/ERK Kinase), and MPK-1 (MAP Kinase) (Chang et al., 2000; Clark et al., 1992; Han et al., 1990; Han et al., 1993; Kornfeld et al., 1995; Lackner et al., 1994; Wu and Han, 1994; Wu et al., 1995). Reduction-of-function (rf) mutations in any of the signaling proteins in the LET-23 RTK pathway result in less than three VPCs undergoing vulval differentiation. In the most severe cases, the disruption of the LET-23-mediated signaling results in the complete failure to generate vulval tissue (vulvaless, or Vul, phenotype). In contrast, an abnormal increase in signaling due to activating mutations in the pathway or the removal of two or more negative regulators of the pathway causes the opposite effect in which greater than wild type numbers of VPCs differentiate into vulval tissue (multi-vulva, or Muv, phenotype).

The *sli-1* locus was identified in a screen for suppressors of the Vul phenotype associated with *let-23(rf)* mutations (Jongeward et al., 1995). *sli-1* encodes proteins of 582 and 540 amino acids that are similar to the mammalian proto-oncogene products c-Cbl, Cbl-b, and Cbl-3 (Keane et al., 1999; Keane et al., 1995; Langdon et al., 1989). SLI-1 and c-Cbl proteins are composed of an N-terminal region, followed by a RING finger domain and a proline rich C-terminus (see Figure 3). The N-terminal region (termed SLI-1:N/Cbl-N) contains three interacting domains: a four helix bundle, an EF hand and a divergent SH2

domain (Meng et al., 1999). Genetic analysis has revealed that *sli-1* is a negative regulator of those *let-23* signaling functions that are mediated though RAS activation (Jongeward et al., 1995). Mice lacking c-Cbl have inappropriate ZAP-70 activity in T cells, indicating that c-Cbl plays a negative role in signaling (Murphy et al., 1998). Experiments performed *in vitro* suggest that c-Cbl directly controls down regulation of RTKs. This is dependent upon the SH2 domain which mediates binding to activated receptor followed by phosphorylation of c-Cbl at a site adjacent to the RING finger domain. The RING finger domain is then essential for the last step of catalyzing receptor ubquitination (Joazeiro et al., 1999; Levkowitz et al., 1999; Levkowitz et al., 1998).

Here we show that c-Cbl and SLI-1 are functionally equivalent. Genetic analysis reveals that SLI-1 acts to negatively regulate EGFR signaling upstream of LET-60 RAS, either at the receptor or Grb2/SEM-5 step. We compare *sli-1* function to that of other negative regulators of this pathway, *gap-1* (Hajnal et al., 1997) and *ark-1* (Hopper et al., 2000). We provide evidence that SLI-1 inhibition is partly mediated through an inhibitory tyrosine in the carboxy terminus of the LET-23 EGFR and, in contrast to the ubiquitin ligase activity, this inhibition is not wholly dependent upon the RING finger of SLI-1.

MATERIALS AND METHODS

Strain construction and maintenance

Strains were maintained at 20°C and handled according to Brenner (1974). The following alleles were used for strain construction: for LGI, *unc-38(x20)*; LGII, *dpy-10(e128), let-23(sy1, sy97), unc-4(e120), clr-1(e1745)*; for LGIV, *unc-24(e138), dpy-20(e1282), ark-1(sy247), let-60(sy127, n1876, n2021, n2022, n2034, n2035)* (Beitel et al. 1990; Han et al. 1990); for LGX, *sli-1(sy143), sem-5(n1619, ay73), gap-1(n1691), unc-2(e55)* (Clark et al. 1992a; M. Stern, personal communication). *szT1* is a reciprocal *I;X* translocation used as a balancer for *sem-5* (Fodor and Deak 1985). *DnT1* is a reciprocal *IV;V* translocation used as a balancer for *let-60* (Ferguson and Horvitz 1985). *sDf8* is a small deficiency in *LGIV* which uncovers *let-60* (Rogalski et al. 1982).

sli-1 sem-5 double mutants were constructed in the following manner: For *sli-1 sem-5(n1619)*, N2 males were mated into *unc-4; sli-1* hermaphrodites. Non-Unc male progeny were selected and mated into *clr-1(e1745); sem-5(n1619)* hermaphrodites. non-Egl (egg laying positive) healthy progeny were picked singly on to individual plates and checked for non-Clr Unc Egl progeny. These non-Clr Unc Egl progeny were again picked singly on to individual plates. If *sli-1* suppressed *sem-5*, then only those recombinants of the genotype *unc-4; sli-1 sem-5(n1619)/+ sem-5(n1619)* would survive to give F2 progeny as *sem-5(n1619)* homozygotes do not propagate beyond F1 from heterozygous mothers. Otherwise, no Unc Egl progeny would survive (except the small number of cases where there was a *clr-1 unc-4* recombinant chromosome; these were selected against by checking for the lack of a Clr phenotype). *unc-4* was removed from *sli-1 sem-5(n1619)* by mating N2 males and selecting non-Unc F1 progeny. Non-Unc, sickly, slow-growing progeny were individually isolated in F2 and again in F3 and checked for non-Unc F4 progeny. For the construction of *sli-1 sem-5(ay73)*, N2 males

were mated into *sli-1* hermaphrodites. Resulting male progeny were mated into *unc-38(x20)/szT1; sem-5(ay73)/szT1* hermaphrodites. Many healthy progeny were picked singly on to individual plates from this cross. F2 notch-heads (an indication of the presence of *sli-1* were then picked singly from only those F1 plates which did not segregate phenotypically long males (*szT1* segregates *lon-2* males). The notch-head F2's could be categorized into three classes depending on their F3 progeny. The first class yielded only healthy progeny (F2 homozygous for *sli-1*. The second class yielded healthy progeny, no dead larvae, no dead eggs, and many Egl animals with dead larvae or dead eggs inside (*sli-1* almost completely suppresses the F1 lethality caused by *sem-5(ay73)* mutation). These F2s were likely to be *sli-1 sem-5(ay73)/sli-1* + recombinants. The third class produced no progeny and were Egl by themselves. This final class of F2s were *sli-1 sem-5(ay73)*. Since *sli-1 sem-5(ay73)* strain can not be maintained as homozygotes, szT1 was used to balance *sli-1 sem-5(ay73)*. The presence of *sli-1* mutation in Egl animals was further confirmed by sequencing.

To construct *sli-1 gap-1* double mutants, *sli-1* males were mated into *gap-1 unc-2* hermaphrodites. Non-Unc cross progeny were individually isolated and checked for Unc progeny. These Unc progeny were then picked singly. Only those recombinants of the genotype *sli-1 gap-1 unc-2/ + gap-1 unc-2* can segregate Muv progeny if *sli-1 and gap-1* synergize to confer the excessive vulval differentiation. On rare plates, Muv progeny were observed and picked singly in the next generation. In their progeny, the low penetrance of notch-head phenotype was used to confirm the presence of *sli-1* mutation.

The *let-60; sli-1* strains were constructed as follows: N2 males were mated into *unc-24(e138) let-60(rf or null)/DnT1; +/DnT1* strains. *let-60(rf)* alleles used were mentioned above. Non-Unc cross progeny males were selected and mated into *dpy-20; sli-1* hermaphrodites. Non-Dpy cross progeny males were then picked and mated into

dpy-20; sli-1. Non-Dpy hermaphrodites were then picked singly. From those F1 plates that yielded Unc progeny, Unc non-Dpy and non-Unc non-Dpy progeny were individually isolated. Since *sli-1* did not suppress *let-60* lethality, non-recombinant Unc non-Dpy progeny did not survive past F1. The strains were maintained as non-Unc non-Dpy heterozygotes of the following genotype: *unc-24(e138) let-60(rf or null) +/+ + dpy-20; sli-1*. F1 Unc non-Dpy progeny were then picked singly and scored for vulval differentiation. Each scored worm was recovered from the slide and allowed to propagate to check for F2 progeny. Vulval differentiation scores from those that gave viable F2 progeny yielding a proportion of Dpy animals were discarded as they represented recombinants that were not homozygous for *let-60* .

To construct the hetero-allelic series, the strain *dpy-10(e128); unc-34(e138) let-60(n2021)/DnT1; +/DnT1* was made by standard methods. *let-60(n2021)* is a weak reduction of function allele. To generate the various hetero-allelic worms, N2 males were mated into the *unc-24(e138) let-60(rf or null)/DnT1; +/DnT1* strains carrying the different severe *let-60* mutations. Non-Unc cross progeny males were selected and mated into the *dpy-10(e128); unc-34(e138) let-60(n2021)/DnT1; +/DnT1* strain. Unc-24, non-Dpy, non-DnT1 F1 hermaphrodites from these crosses were scored for vulval differentiation. Scored worms were recovered and their progeny were checked to ensure that *let-60(rf or null)* remained homozygous.

Other strains were constructed following standard methods.

An in vivo transgenic assay for the activity of sli-1 minigenes

To establish a system in which to study the relationship between SLI-1 structure and its function, we constructed *sli-1* cDNA minigene constructs driven by the heat shock promoter *hsp16-41* (Mello and Fire, 1995; Stringham et al., 1992). To facilitate expression, an artificial intron is present between the promoter and the cDNA insert.

Heat shock promoters were used in these experiments because initial tests using genomic sequences 5' to the start codon of *sli-1* fused to the full-length *sli-1* cDNA failed to confer significant wild-type SLI-1(+) activity in the vulva (data not shown), presumably because sequences present within the introns of *sli-1* are necessary for expression.

Germline transformation was performed according to the methods of Mello et al. (1991). Heat shock constructs were injected at 50 ng/µl along with 150 ng/µl of SK+ plasmid and 15 ng/µl of pMH86 (*dpy-20(+)* marker) into the germline of *let-23(sy1); dpy-20; sli-1* animals. Independent non-Dpy transformant lines were maintained and selected for transgene stability. F3 stable transgenic progeny were selected for egg-lay cohorts and heat shock analysis. For the egg-lay cohorts, we selected only those worms with a egg-laying rate similar to that of wild-type hermaphrodites (≈ 5-10 eggs laid per worm per hour).

Thirty egg laying young hermaphrodites were moved onto fresh agar plates, ten worms per plate. These were then allowed to lay eggs on given plates for one hour before being moved to fresh plates. The hourly transfer of worms was continued for six consecutive hours and the egg-laying hermaphrodites were then removed from the plates. The plates were maintained at 20 °C during and after establishment of egg cohorts. 36 hours after the hermaphrodites' removal from the final cohort of eggs, the plates were heat shocked for 30 minutes at 33 °C. We thus generated worms synchronized in age into hourly cohorts that had been heat shocked between 36 and 42 hours after egg lay. This interval encompasses the early L3 stage during which vulval induction occurs in the transgenic animals. The heat shocked plates were returned to 20 °C for 18 to 24 hours following heat shock.

Vulval differentiation was scored in non-Dpy progeny at early to mid L4 stages. A minimum of two independent lines were scored per heat shock construct. 15 to 30 worms were scored per one hour time interval per stable line following heat shock. The

distributions generated from each heat shock construct (as seen in the histograms of Figure 4B) were entered into the InStat program (GraphPad Software, version 2.0) and compared in pairs using the Mann-Whitney test to generate two-tailed P values. There is ~2 cell range in the average induction levels between the no insert control and the full-length *sli-1* cDNA construct. This range allows comparisons of the various induction levels in different minigene constructs. In the structure-function experiments, SLI-1activity is inferred in those minigene constructs that lower average vulval differentiation to significantly below 2.5 vulval cells/worm.

sli-1 minigenes and mutagenized let-23 constructs

Standard molecular biological techniques were used as described by Sambrook et al. (1989). Site directed *in vitro* mutagenesis was carried out in double stranded DNA according to the method prescribed by Deng and Nickoloff (1992) and with reagents and specific protocols from Clonetech (Palo Alto, CA). The mutagenesis was carried out in the plasmid pCY-D6, a pSK+ vector containing the full-length *sli-1* cDNA inserted into the EcoRI site. A selection primer which changed the novel NotI site in the vector to an NheI site was used in conjuction with mutagenic primers which mutated or removed sequences of varying length within the *sli-1* coding region. Mutagenized *sli-1* cDNA was then digested with SpeI/EcoRV and inserted into the NheI/EcoRV sites of the pPD49.83 and pPD.49.78 nematode heat shock minigene vectors. Mutagenesis was confirmed by restriction mapping and by sequencing. The deletion constructs SLI-1.ΔNΔRINGΔC, SLI-1.ΔC, SLI-1.ΔNΔRING, SLI-1.ΔPro2, and SLI-1.ΔPro3 replace the deleted sequences with an NheI site which codes for alanine and serine in frame with the remainder of the *sli-1* construct. Primers used for *in vitro* mutagenesis of the *sli-1* cDNA are as follows: SKNotNhe; Del-NC(Nhe); Del-C(Nhe); Del-N(Nhe); Altspli; Del-PRO2(Nhe); Del-PRO3(Nhe); Del-RING.

For the deletion of the putative myristylation site, we utilized a selection primer which changed a unique XhoI site into a ClaI site in the various mutagenized *sli-1* cDNAs. Primers used were SKXhoCla and Del-MYR1. The cloning into heat shock vectors followed identical procedures as above.

The conserved N-terminal domain of human c-Cbl was PCR amplified from the cDNA in a pUC vector (provided by W. Langdon) using the primers hCbl-N1A(Nhe) and hCbl-N2R(Nhe). The ends of the amplification primers contained in-frame NheI sites. The amplified conserved N-terminal fragment of c-Cbl was then purified, digested and ligated into the NheI site of the SLI-1.ΔN construct in pPD49.83. The directionality of the insert was checked by restriction digests and the sequence of the amplified c-Cbl fragment was verified by DNA sequencing. DNA sequencing was carried out on automated sequencers (Applied Biosystems) by the Caltech DNA Sequencing Facility.

All the systematically mutagenized *let-23* constructs were made as described by Lesa and Sternberg (1997). Engineered *let-23* constructs were injected into the germline of *let-23(sy17)unc-4/mnc1; dpy-20; sli-1* or *let-23(sy17)unc-4/mnc1; dpy-20 ark-1* mothers; the Unc-4, non-Dpy stable transformed progeny (F2 or later generation) were tested for effects in vulval differentiation.

Primer designations

Primer names and their corresponding sequences used during *in vitro* mutagenesis are as follows:

SKNotNhe:5'- ACCGCGGTGGCTAGCGCTCTAGAAC

Del-NC(Nhe):5'- GCCCGGTTTCTGCAGTGAAGAGGCTAGCTAGACTTGTGTAAATGTTCATCTTACC

Del-C(Nhe):5'- GTGTGATTATTGACAGGTTCAAGCCCGCTAGCTAGACTTGTGTAAATGTTCATCTTACCG

Del-N(Nhe):5'- GATGCCCGGTTTCTGCAGTGAAGAGGCTAGCACTCCGGTAGAAATTGAAAAAGCG

Altspli:5'- CCCGACGTGCCTCCCAGAACGTCGTCACAAACATCCTCTTCATACG

Del-PRO2(Nhe):5'- CTCAATTCCGTCGGTCGACGAGGCTAGCGCATTGGGTACCCTGGACAC

Del-PRO3(Nhe):5'- GGCACAAGTGGTAAACCGGCAACGGGCTAGCTCAGCGAGCGAGCACCAACCACACC

DEL-RING:5'- TTGTGAGATGGGCACAACATTCGAGTACGAAATCAAAGGAACAAATCGTGT

SKXhoCla:5'- ACCGTCGACATCGATGGGGGGCCCG

Del-MYR1:5'- GTTTCACCGGGAATGGCTAGCATAAACACAATTTTTC

hCbl-N2R(Nhe):5'- GCCACTGCTAGCAGGATCAAACGGATCTACCAC

hCbl-N1A(Nhe):5'- CCGCCGGGGGCTAGCGACAAGAAGATGGTGGAG

Microscopy

The extent of vulval differentiation was measured by examining vulval anatomy in early to mid L4 stage animals (Sulston and Horvitz 1977; Sternberg and Horvitz 1986). Hermaphrodites were placed on 5 % Noble Agar pads and scored with a Plan 100x objective, Nomarski differential interference-contrast optics. 1° and 2° vulval fates were scored as vulval cells. Wild-type=3 VPCs undergoing vulval differentiation/worm=100 % vulval differentiation.

RESULTS

sli-1(lf) suppresses a severe reduction of function allele but not a null allele of sem-5

Mutations in *sem-5* result in a partial or complete lack of a vulva. *sem-5* encodes an SH3-SH2-SH3 domain containing Grb2 homolog that is required to transduce the signal from LET-23 to LET-60 Ras (Clark et al., 1992; Clark et al., 1992; Katz et al., 1996; Lesa and Sternberg, 1997; Stern et al., 1993). *sem-5(ay73)* is an early missense mutation, Q10amber (Q at codon 10 mutated into amber stop), that removes all of the functional

domains of the SEM-5 protein and is therefore a putative null allele (M. Stern, personal communication). Animals homozygous for *ay73* are inviable in the F2 generation, with F1 escapers (due to maternal rescue) being completely vulvaless (Table 1). The *sem-5(n1619)* allele generates a P49L substitution which most likely results in a non-functional N-terminal SH3 domain (Clark et al., 1992). Animals homozygous for *n1619* are also inviable in the F2 and viable F1 animals have, on average, only 0.4 vulval cells induced/animal. In wild-type animals 3 cells are invariably induced to form the vulva.

We constructed *sli-1 sem-5* double mutants using the *sli-1* reference allele, *sy143*. The molecular lesion associated with *sy143* is Q152amber, which removes the carboxyl-terminal 75% of the SLI-1 protein. In addition, it is the most severe allele and likely generates a non-functional product (Jongeward et al., 1995; Yoon et al., 1995). *sli-1* strongly suppresses the vulval defect in *sem-5(n1619)* mutants: *sli-1 sem-5(n1619)* have near wild-type levels of vulval differentiation (average 3.0 vulval cells/worm; not all animals have wild type level of vulval induction; Table 1, Figure 2d). The lethality of the *sem-5(n1619)* allele is also partially suppressed: double mutants can be maintained as homozygotes. In contrast, *sli-1* does not suppress the *sem-5(ay73)* null allele: *sli-1 sem-5(ay73)* double mutants are Vul when scored as F1 homozygotes and can not be maintained as homozygotes beyond F2 from heterozygous mothers (Table 1, Figure 2f). However, there is increased survival of *sli-1 sem-5(ay73)* F1 homozygotes from heterozygous mothers in comparison to *sem-5(ay73)* single mutant F1 homozygotes. Thus, mutations in *sli-1* can enhance maternal rescue of lethality associated with *sem-5*, consistent with the absence of *sli-1* compensating for a reduction in *sem-5* activity.

sli-1(lf) does not suppress severe reduction of function alleles of let-60

We tested three *let-60* alleles for suppression by *sli-1*. These *let-60* alleles, *n1876* (Ala66Thr), *n2034* (Ala66Thr) and *n2035* (Ala66Val), are described by Beitel et al. (1990);

corresponding mutations have been made in Ha-Ras and shown to interfere with exchange factor interaction (Boriack-Sjodin et al., 1998; Howe and Marshall, 1993). We find that *sli-1* fails to suppress the vulvaless phenotype of these alleles (Table 1). In addition, *sli-1* also fails to suppress the lethality associated with these three *let-60* alleles. However, as in the case with *sem-5(ay73)*, there is an increase in F1 homozygote survival from *let-60(rf)/+* heterozygous parents in a *sli-1* background, indicating enhanced maternal rescue of lethality (data not shown).

sli-1 fails to suppress null alleles of *let-23* and *sem-5* while it strongly suppresses severe reduction of function alleles of *let-23* and *sem-5* (Jongeward et al., 1995; this report). It was therefore crucial to determine whether the tested *let-60* alleles, *n1876*, *n2034* and *n2035*, were non-null alleles. To distinguish between severe non-null alleles and null *let-60* alleles, we measured the extent of vulval differentiation in a series of F1 trans-hetero-allelic animals using a weak reduction of function *let-60* allele (*n2021*), *in trans* to the three *let-60* alleles (*n1876*, *n2034* and *n2035*), as well as a putative *let-60* null allele (*sy127*) and *sDf8*, a deficiency which uncovers the *let-60* locus as controls. *let-60(n2021)* is also an exchange factor defective mutant (G75S) (Howe and Marshall, 1993). If *n1876*, *n2034* and *n2035* are non-null mutations then *in trans* to *let-60(n2021)* should be less severe than *let-60(null)/let-60(n2021)* heterozygote controls in a vulval induction assay. In these experiments, *n2035* and *n1876* had significantly more activity than the *sy127* null allele and the *sDf8* deficiency in the vulva (1.6 and 1.4 cells versus 0.8 and 0.6 cells being induced on average (n>22)). Therefore, *n1876* and *n2035* are likely to be reduction of function alleles. Thus, *sli-1(rf)* fails to suppress non-null *let-60(rf)* mutations in the vulva. Since lack of *sli-1* activity can compensate for decreased function of *let-23* and *sem-5*, but not *let-60*, we conclude that SLI-1 acts upstream of RAS activation.

Mutations in sli-1 give a Muv phenotype in a gap-1 mutant background

GAP-1 is a negative regulator of vulval signaling that likely acts at the level of LET-60 Ras (Hajnal et al., 1997). A *gap-1(lf)* reference allele suppresses the Vul phenotype of *let-60(n1876)* while only weakly suppressing the *sem-5(n2019)* mutation (Hajnal et al., 1997). As in the case with *sli-1* single mutants, *gap-1* mutations by themselves are silent.

We constructed *sli-1 gap-1* double mutants. This strain has a partially penetrant Muv phenotype, with 7 of 24 observed double mutants exhibiting an excessive vulval induction (Table 1). *gap-1* corresponds to a Q149ochre nonsense mutation which results in early truncation and presumably defines a null allele (Hajnal et al., 1997). The simplest interpretation of this result is that SLI-1 and GAP-1 have, at least partially, distinct activities.

The conserved N-terminal region and RING finger domain rather than the proline-rich C-terminal region is sufficient for the inhibitory function of SLI-1

We established an *in vivo* transgenic assay for the activity of *sli-1* minigenes being expressed from the *hsp16-41* heat-shock promoter in the genetic background of *let-23(sy1); sli-1* (see methods). The vulvaless phenotype of *let-23(sy1)* animals is strongly suppressed by *sli-1* (see table 1). In control transgenic animals of genotype *let-23(sy1); dpy-20; sli-1; syEx*[dpy-20(+); hsp16-41], heat-shocked at the time of vulval induction, the average vulval induction is 2.5 cells/worm (Figure 4). Under the same experimental conditions and genetic background, the full-length *sli-1* cDNA driven by the *hsp16-41* promoter confers full SLI-1(+) activity with animals having on average 0.5 VPCs executing a vulval fate per worm (henceforth referred to as vulval cells/worm; Figure 4). This is a full 2 cells lower than the vulval induction seen in the no insert control and is comparable to the levels of vulval differentiation seen in *let-23(sy1)* single mutants in a non-Dpy-20 germline transformed background (0.3 vulval cells/worm) (Yoon et al., 1995).

In this transgenic assay, SLI-1 proteins lacking either the second or third proline rich domain retained full activity (average induction 0.7 and 0.3 vulval cells/animal; P=0.14 and 0.39 respectively). Likewise, expression of the alternatively spliced *sli-1* cDNA which deletes the 10th exon of the genomic sequence has activity similar to that of full length SLI-1 (average 0.9 vulval cells/worm; P=0.05; Figure 4A). Moreover, we found that expression of a SLI-1 protein truncated after residue 447 and thus lacking the entire proline-rich C-terminal domains also retains significant SLI-1(+) activity (average 1.0 vulval cells/worm; P<0.0001 versus no insert control; Figure 4A, B). However, this level of activity is also significantly weaker than that of the full-length *sli-1* cDNA (P=0.0002). To test whether proline-rich C-terminal domains are partially sufficient for SLI-1 function, a *sli-1* construct lacking the N-terminal region and RING finger domain (deleting residues 58 to 447) was tested; it does not have SLI-1(+) activity under identical assay conditions (vulval induction levels similar to the no insert control, P=0.8; Figure 4A, B).

Consensus myristylation site is not required for SLI-1(+) activity

In the conceptual translation of SLI-1, the initiator methionine is followed in sequence by Gly-Ser-Ile-Asn-Thr, a myristylation site (reviewed by Resh 1994). Myristylation may play a role in localizing proteins to the cell membrane, usually in combination with other lipid modifications or nearby basic residues (Resh, 1994). Using site directed mutagenesis, we substituted the second codon glycine with alanine (G2A substitution); alanine should maintain the charge neutral hydrophobic character of glycine but prevent the covalent addition of myristate at the second codon. Constructs expressing the full-length and truncated SLI-1 proteins with G2A substitutions do not have less activity than control constructs (Figure 4A; P>0.2). In addition, the SLI-1 construct that lacks the N-terminal conserved SH2 and RING finger domains has no activity in the

vulva after the G2A substitution (Figure 4A). As mentioned previously, the first ~50 amino acid residues of c-Cbl are highly divergent in sequence from those of SLI-1 and the consensus myristylation sequence is conserved neither in c-Cbl, Cbl-b or Cbl-3.

The conserved N-terminal region of c-Cbl containing the SH2 and RING finger domains can functionally replace that of SLI-1

The human c-Cbl protein is 906 amino acid residues in length. The ~400 amino acid stretch of high sequence similarity (~55 % identity) between c-Cbl and SLI-1 begins after a stretch of ~50 N-terminal residues in the two proteins (Figure 3). This region contains the SH2 and RING finger domains strongly implicated in Cbl function (Joazeiro et al., 1999; Levkowitz et al., 1999). The N-terminal 45 and 58 residues of c-Cbl and SLI-1, respectively, are highly divergent in sequence. Furthermore, although both proteins have several putative SH3-binding polyproline motifs in their respective C-terminal domains, the amino acid sequence following residue 447 in SLI-1 and residue 437 in c-Cbl are also largely divergent.

To test for the functional similarity between the conserved domains of SLI-1 and c-Cbl, we constructed a chimeric *sli-1/c-cbl* cDNA by replacing the coding sequence of SLI-1's N-terminal region and RING finger domain (residues 58 to 447) with that of c-Cbl (residues 45 to 437). We find that this c-Cbl/SLI-1 chimeric construct driven by the *hsp16-41* promoter exhibits partial SLI-1(+) activity (1.5 vulval cells/worm; $P<0.0001$ versus both no insert control and the full-length *sli-1* cDNA; Figure 4A, B). As a control, a *sli-1* construct lacking the N-terminal region and RING finger domain (deleting residues 58 to 447) was tested; it was shown not to have SLI-1(+) activity under identical assay conditions (vulval induction levels similar to the no insert control, $P=0.8$; Figure 4A, B). However, the full-length c-Cbl cDNA driven by the *hsp16-41* promoter does not have SLI-1(+) activity in the vulva (data not shown).

A SLI-1 protein lacking the RING finger domain retains some activity

Both *sli-1* and *c-Cbl* function as negative regulators of signaling. c-Cbl has recently been shown to have a ubiquitin ligase activity that is proposed to explain the negative regulation of RTKs signaling observed (Joazeiro et al., 1999; Levkowitz et al., 1999). Thus, the SH2 domain presumably mediates binding to activated receptor, and the RING finger domain then catalyzes receptor ubiquitination. The SH2 and RING finger domains of c-Cbl share 55% identity with those of SLI-1 and can functionally replace them in our transgenic assay. We sought to test whether the RING finger domain of SLI-1 was essential for activity. We found that significant SLI-1(+) activity is observed in constructs lacking the RING finger (average 1.5 vulval cells/worm; $P<0.0001$ versus no insert control; Figure 4A, B). However, in comparison to the full length cDNA, the RING deletion significantly reduces the SLI-1(+) activity ($P<0.0001$). Thus, although the RING finger domain is important for SLI-1 activity, a ubiquitin ligase function for SLI-1 is not the sole inhibitory function of *sli-1*.

pYKTEP defines an inhibitory site in the C-terminal tail of LET-23, mediating the negative effect of SLI-1

We next explored the possibility that SLI-1 exerts its inhibitory effect by direct or indirect binding to specific pTyr sites in LET-23. The *let-23(sy97)* mutation deletes the last 56 amino acids of the receptor which include the only three tyrosines (pTyr site 6, 7 and 8) in the receptor carboxy terminal tail which, if phosphorylated, would create SH2 binding sites matching the consensus binding site for the SEM-5 SH2 domain. *sli-1(lf)* strongly suppresses *let-23(sy97)* which suggests that pTyr site 1 through pTyr site 5 could mediate, directly or indirectly, negative regulation of *let-23* signaling by *sli-1*

under the above hypothesis. We introduced engineered *let-23* constructs, with certain codons specifying LET-23 carboxy-terminal tyrosine residues mutated to phenylalanine codons, into nematodes with a *let-23(null)* background with or without a *sli-1* or *ark-1* mutation and assayed their activity by scoring transgenic animals for vulval differentiation. Our results suggest that SLI-1 function is dependent upon LET-23 carboxy-terminal tyrosine residues. Particularly, we observed that the inhibition of signaling by the presence of pTyr2 could be overcome by mutations in *sli-1* (Table 2). We therefore sought to determine whether the absence of this site would synergize with mutations in genes known to negatively regulate *let-23* signaling. We did not observe any synergy between *let-23(null); syEx*[LET-23 ($Y2^-$)] and *sli-1*. However, we observed that *let-23(null); syEx*[LET-23 ($Y2^-$)] did synergize with *ark-1* resulting in 34% of animals having greater than wild-type levels of vulval induction (Table 2). This synergy was not observed in *ark-1* animals over-expressing an intact LET-23 construct (table2). *ark-1* was isolated as an enhancer of *sli-1* and encodes a non-receptor tyrosine kinase related to Ack that inhibits LET-23 signaling (Hopper et al., 2000). 40% of *ark-1; sli-1* double mutant animals have a hyperinduced vulval phenotype with the extra induction usually being in P4.p (Table 3). This phenotype is distinct from *ark-1; gap-1* double mutant animals which have a higher penetrance of extra induction, usually as a consequence of P8.p induction (Table 3). It is striking that the hyperinduced vulval phenotype of *let-23(null); ark-1; syEx*[LET-23 ($Y2^-$)] is nearly identical to that seen in *ark-1; sli-1* animals, both in penetrance and frequency of P4.p induction versus P8.p induction (Table 3).Together, this lead us to conclude that pTyr 2 (pYKTEP) mediates its effects through *sli-1*. This conclusion is strengthened by a recent solved crystallographic structure of c-Cbl complexed to a tyrosine-phosphorylated inhibitory site of protein tyrosine kinase ZAP-70 (Meng et al., 1999). The interaction is mediated by a divergent SH2 domain of c-Cbl, which is conserved in SLI-1, and a phosphotyrosine of ZAP-70, which is in a similar amino acid context to the one in LET-23 we identified (pYXXEP

versus pYKTEP; XX represents pY+1 and pY+2 residues which are not in the interface of the binding complex).

DISCUSSION

The *sli-1* locus was originally defined by mutations that suppress partially defective LET-23, a *C. elegans* homolog of EGF-receptor subfamily tyrosine kinases (Jongeward et al., 1995). The overall sequence of SLI-1 resembles that of the mammalian proto-oncoprotein c-Cbl and related proteins (Cbl-b and Cbl-3). In this study we have found that: 1) Mutations in SLI-1 suppress reduction-of-function mutations in SEM-5 but not LET-60 RAS mutations that likely interfere with exchange factor interaction. 2) Mutations in SLI-1 do not bypass the requirement for SEM-5: a *sem-5* null allele is not suppressed by *sli-1*. 3) The SLI-1 N-terminal region and RING finger domain is sufficient to confer partial wild-type SLI-1 activity in the vulva. 4) The conserved N-terminal region and RING finger domain in c-Cbl can substitute for that of SLI-1 such that a chimeric protein can negatively regulate vulval differentiation. 5) The ubiquitin ligase domain of SLI-1 is not absolutely required for negative regulation by SLI-1.

Our genetic analyses led us to believe that SLI-1 inhibits signaling between LET-23 and RAS for the following reasons. Reduced activity of SLI-1 can increase vulval signaling in a *sem-5(rf)* background: *sli-1*, the reference allele, strongly suppresses the vulvaless phenotype of non-null *sem-5* reduction of function alleles (Jongeward et al., 1995); (this report). However, we have also shown that *sli-1* does not suppress a *sem-5* null allele. Thus, SEM-5 activity is essential for RTK-mediated vulval signaling with or without the presence of wild-type SLI-1. We conclude that SLI-1 is a negative regulator of SEM-5-dependent signaling following LET-23 activation. In contrast, severe reduction of LET-60 Ras activity is not compensated for by the absence of SLI-1. We thus infer that SLI-1 affects vulval signaling upstream of LET-60 Ras. Furthermore, the

Muv phenotype of *sli-1 gap-1* double mutant animals indicates that SLI-1 and GAP-1 define, at least partly, independent pathways. Recently, another GAP gene, *gap-2*, has been identified in *C. elegans* (Hayashizaki et al., 1998); however, mutations of *gap-2* do not suppress the vulvaless phenotype of *let-23* mutations (Hayashizaki et al., 1998); (C. Chang, unpublished observations). *gap-1* and *gap-2* are the only RasGAPs found in *C. elegans* by genome-wide blast search.

Results from our SLI-1/c-Cbl chimeric construct indicate that the N-terminal region and RING finger domains of SLI-1 and c-Cbl are functionally conserved with respect to vulval signaling. Our structure-function studies show that the conserved N-terminal region and RING finger domain of SLI-1 are necessary and almost sufficient for negative regulatory activity in LET-23-mediated signal transduction. That the N-terminal region and RING finger domain may have a conserved regulatory function in RTK signaling is supported by two lines of evidence from elsewhere: 1) Identification of a *Drosophila* c-*cbl* homolog (*Drosophila-Cbl*; or *D-Cbl*) revealed that the D-Cbl product shares the conserved N-terminal domain with c-Cbl and SLI-1 (Hime et al., 1997; Meisner et al., 1997). As in the alignment of SLI-1 and c-Cbl, the sequence similarity of D-Cbl begins after a stretch of divergent N-terminal residues that are different from both c-Cbl and SLI-1. However, unlike SLI-1 and c-Cbl, the D-Cbl sequence ends shortly C-terminal to the RING finger motif and contains no polyproline motifs. As expected from the lack of poly-proline motifs, D-Cbl does not bind Drk, the *Drosophila* homolog of SEM-5/Grb2 adaptor (Hime et al., 1997; Meisner et al., 1997). Furthermore, both reports show that D-Cbl associates with the *Drosophila* EGF receptor in an activation-dependent manner. Finally, Meisner et al. (1997) show that the expression of D-Cbl under the *sevenless* enhancer in *Drosophila* negatively regulates R7 photoreceptor development. These results suggest that the conserved N-terminal domains of SLI-1 and D-Cbl play analogous roles and that these domains are sufficient for the negative regulation of the LET-23 and *Drosophila* Sevenless pathways. 2) It was previously

shown that introduction of a hypomorphic Gly to Glu missense mutation found in SLI-1's N-terminal domain (Yoon et al., 1995) can abolish c-Cbl binding to ZAP-70 and EGFR and ablate the transforming function of v-Cbl (Lupher et al., 1996; Thien and Langdon, 1997). Thus, identical mutations in a conserved residue in the N-terminal domains disrupts function in both SLI-1 and c-Cbl, suggesting structural and functional conservation.

It is unclear why the full length c-Cbl construct does not have SLI-1(+) function in our assays despite the fact that the conserved N-terminal domain of c-Cbl can functionally replace the corresponding domain in SLI-1 in a chimeric construct. Several explanations are possible. One reason may be that the size of c-Cbl's C-terminal domain prevents sterically an effective association with the cytoplasmic portion of the LET-23 protein: the C-terminal domain of c-Cbl is three times the mass of that of SLI-1. Or, the nematode translational machinery does not properly recognize the initiator methionine of c-Cbl, therefore preventing effective expression. A third possibility is that the highly divergent stretch of residues N-terminal to the conserved domains could serve important species-specific functions.

The C-terminal polyproline motifs, though not essential for SLI-1 function, are necessary for the full wild-type negative regulatory activity: the SLI-1:N+RING finger protein is significantly less effective than the full length SLI-1 (39% Vul for the SLI-1:N+RING finger protein vs. 74% Vul for full-length SLI-1, Figure 4B; P=0.0002). This effect may simply be caused by a reduction in protein stability due to early truncation. However, we have found that the polyproline rich C-terminus of SLI-1 can interact with SEM-5 in a yeast 2 hybrid assay (in this study and Walhout et al., 2000). This raises another possibility for the function of the C-terminal polyproline motifs: SEM-5, or a similar adaptor, may bind to SLI-1 and increase the efficacy of SLI-1 localization to the RTK complex, due to the binding of the adaptor to the receptor pTyr sites. In mammalian cells, c-Cbl's C-terminal polyproline domains have been shown to bind

adaptors such as Grb2 and Nck via PPII helix-SH3 interactions (Clements et al., 1999; Donovan et al., 1996; Meisner and Czech, 1995; Rivero-Lezcano et al., 1994). Furthermore, it has been shown that Grb2 can mediate an indirect association between c-Cbl and EGFR (Meisner and Czech, 1995). In a similar manner, the SLI-1's C-terminal polyproline domains may bind to SH3 domains of adaptor proteins which in turn enhance the localization of SLI-1's N-terminal domain to the activated LET-23 receptor. In addition, it is also possible that SLI-1's polyproline domains may compete with the polyproline domains of Sos in binding SEM-5, thereby enhancing SLI-1's inhibition of the LET-23 RTK pathway.

The RING finger domain of c-Cbl has been recently shown to enhance ubiquitination of active RTKs by acting as an E3 ubiquitin-protein ligase (Joazeiro et al., 1999; Levkowitz et al., 1999; Levkowitz et al., 1998). Thus, the effects of loss of *sli-1* activity might be explained by the failure of the LET-23 RTK to be down-regulated by a ubiquitination dependent degradation pathway. However, we find that the RING finger domain of SLI-1 is partially dispensable: a SLI-1 variant lacking its RING finger domain retains a significant amount of biological activity. Thus the ubiquitin-ligase activity of SLI-1/c-Cbl is unlikely to be the sole activity of SLI-1. This conclusion is strengthened by other findings. It might be expected that if the sole function of SLI-1 was to target activated receptor for degradation that a) there might be more LET-23 observable in a *sli-1(lf)* background and b) that over expression of LET-23 might mimic *sli-1(lf)*. Neither of these effects have been observed (Table 2).

In summary, we find that SLI-1 inhibits LET-60 RAS activation by LET-23. We suggest that SLI-1 interacts with the LET-23 signaling complex via at least two domains. One of these domains, the proline-rich C-terminal portion, is necessary for the wild-type level of activity of SLI-1 in the context of a full-length protein; its role is likely to interact with a SH3 domain-containing protein. SEM-5 is a candidate due to the interaction between SLI-1 and SEM-5 we observe in our yeast two-hybrid assays. Since

the C-terminal proline-rich domain alone is not sufficient to inhibit signaling, we infer that SLI-1 does not simply titrate SEM-5 from the RTK/Ras pathway. In addition, a SLI-1 protein lacking this proline rich C-terminal portion retains inhibitory function on signaling. Thus, the remaining N terminal and RING finger domains must be able to interact with some of the components of the signaling complex directly by a different mechanism. In mammalian cell lines, the conserved N-terminal domain of c-Cbl associates directly with the autophosphorylated C-terminal region of the EGF receptor (Bowtell and Langdon, 1995; Galisteo et al., 1995; Lupher et al., 1997). The N-terminal domain has also been shown to associate with the non-receptor tyrosine kinase ZAP-70 in a phosphorylation-dependent manner in T cells (Lupher et al. 1996). In both cases, the association requires the N-terminal divergent SH2 domain, and not the C-terminal polyproline motifs. We explored the possibility that SLI-1 may exert its inhibitory effect by direct or indirect binding to specific pTyr sites in LET-23. By analyzing the systematically mutagenized *let-23* constructs containing substitutions in the carboxyl-terminal tyrosine residues, we have identified an inhibitory tyrosine residue, that can overcome the negative regulation by *sli-1* when it is mutated. Our current models for *sli-1* functions propose two roles (Figure 1). The major role of *sli-1* might be to attenuate signaling after activation has occurred. Upon induction, SLI-1 is recruited into the receptor signaling complex by itself or an adaptor protein and negative regulation ensues by some other means, possibly by preventing the association and activation of downstream effectors such as the SEM-5 adaptor and/or LET-341 SOS-1, and catalysing the ubiquitination of the receptor and thus targeting it for degradation. The minor role of *sli-1* might be to regulate the basal activity of signaling in quiescent state by competing with LET-341 SOS-1 for the binding of SEM-5, thereby decreasing the chance that the spontaneously activated receptor recruits the SOS-1-bound SEM-5 in the absence of ligand. Other models are possible since we can not rule out the possible existence of uncharacterized catalytic domains in SLI-1.

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Table 1.

sli-1 suppression of mutations in *let-23*, *sem-5* and *let-60*

Genotype	Average number of VPCs undergoing vulval differentiation	
	sli-1(+)	*sli-1(sy143)*
+	3.0 (*)	3.0 (n=20)#
let-23(sy1)	0.8 (n=29)#	4.3 (n=30)
let-23(sy97)	0.0 (n=20)#	2.9 (n=131)
sem-5(n2019)	0.5 (n=20)#	2.6 (n=23)#
sem-5(n1619)	0.4 (n=40)	3.0 (n=84)
sem-5(ay73)	0.0 (n=20)	0.02 (n=23)
gap-1(n1691)	3.0 (n=21)	3.2 (n=24)
let-60(n1876)	0.0 (n=62)	0.08 (n=80)
let-60(n2034)	0.02 (n=62)	0.03 (n=87)
let-60(n2035)	0.05 (n=66)	0.09 (n=81)

Legend: Extent of vulval differentiation in *let-23*, *sem-5*, *gap-1* and *let-60* mutants in *sli-1(+)* and *sli-1(-)* backgrounds. The number values represent average number of VPCs

undergoing vulval differentiation per worm; 3.0 is wild-type level. n=number of animals scored. (*), n=many. #, data from Jongeward et al. (1995).

Table 2.

pTyr site 2 in the carboxyl terminal tail of LET-23 mediates negative regulation of LET-23 signaling by SLI-1

	% Average vulval induction[a]		
C-terminus	*ark-1(+); sli-1(+)* [b]	*sli-1(-)*	*ark-1(-)*
1 2 3 4 5 6 7 8	99 (n=22)		91 (n=63) [c]
- - - - - - - -	4 (n=42)		
- 2 - - - - - -	--- [d]	--- [d]	
- - 3 - - - - -	3 (n=11)	8 (n=28)	
- - - 4 - - - -	93 (n=42)	94 (n=8)	
- - - - 5 - - -	11 (n=53)	21 (n=22)	
- - - - - 6 - -	100 (n=65)	89 (n=67)	
- 2 - 4 - - - -	42 (n=85)	86 (n=47)	48 (n=76)
1 - 3 4 5 6 7 8	99 (n=26)	82 (n=41)	107 (n=32)[e]

Legend: Carboxyl-terminal sites 1, 2, 3, etc., indicate that tyrosine(s) at that putative SH2-binding sited has not been changed; a dash indicates that each tyrosine in the corresponding site has been substituted with phenylalanine. At least two stable lines were analyzed per construct, except for the site 3 construct which has only one stable line analyzed.

[a] Average percentage of vulval precursor cells adopting vulval fates per animal.

[b] Taken from Lesa and Sternberg (1997).

[c] One animal from 63 had greater than 3 cells induced (3.5 cell induction).

[d] The presence of site 2 in the absence of other carboxyl-terminal sites confers animals inviability in the genetic backgrounds of *let-23(lf)*. *sli-1(lf)* weakly suppresses this lethality. [e] 34% of scored animals are Muv.

Table 3.

Frequency of induction of the Pn.p cells in different mutants

	Frequency of being induced						Average induction	
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p		n
ark-1; sli-1	1.3%	26.3%	100%	100%	100%	6.3%	3.35	40
ark-1; gap-1	5%	15%	100%	100%	100%	80%	4.0	20
ark-1; Y2⁻	3.2%	25.8%	100%	100%	100%	5.6%	3.35	62

Legend: Animals scored at 20°C. *ark-1; Y2⁻* animals actual genotype is *let-23(null) unc-4; dpy-20 ark-1; syEx[dpy-20(+); let-23(Y2⁻)*. Only those animals with greater or equal to 3 cells induced scored for *ark-1; Y2⁻*. Data pooled from 3 independent lines.

Figure 1.

Models for negative regulation of LET-23 EGFR signaling by SLI-1. The interpretation is based on our molecular genetic analyses presented here or biochemical assays of others presented in other model systems. LIN-3 binding to the extracellular domain induces LET-23 dimerization and autophosphorylation of tyrosine residues that are located in its C-terminal region, including Y2. The latter targets for the SH2 domain of SLI-1. Localization of SLI-1 to the active receptor presumably enables RING finger (RF) domain to recognize the substrates and promote their ligation to ubiquitin via E2 molecule, marking active receptor for degradation. The recruitment of SLI-1 to the active receptor might be helped by the binding of the SEM-5 to the receptor Yx sites. The interaction between SLI-1 and SEM-5, which is mediated by the proline-rich domain of SLI-1 and the SH3 domain of SEM-5, might interfere with the localization of LET-341 to the cell membrane by SEM-5. Yx represents the docking sites in LET-23 for SEM-5.

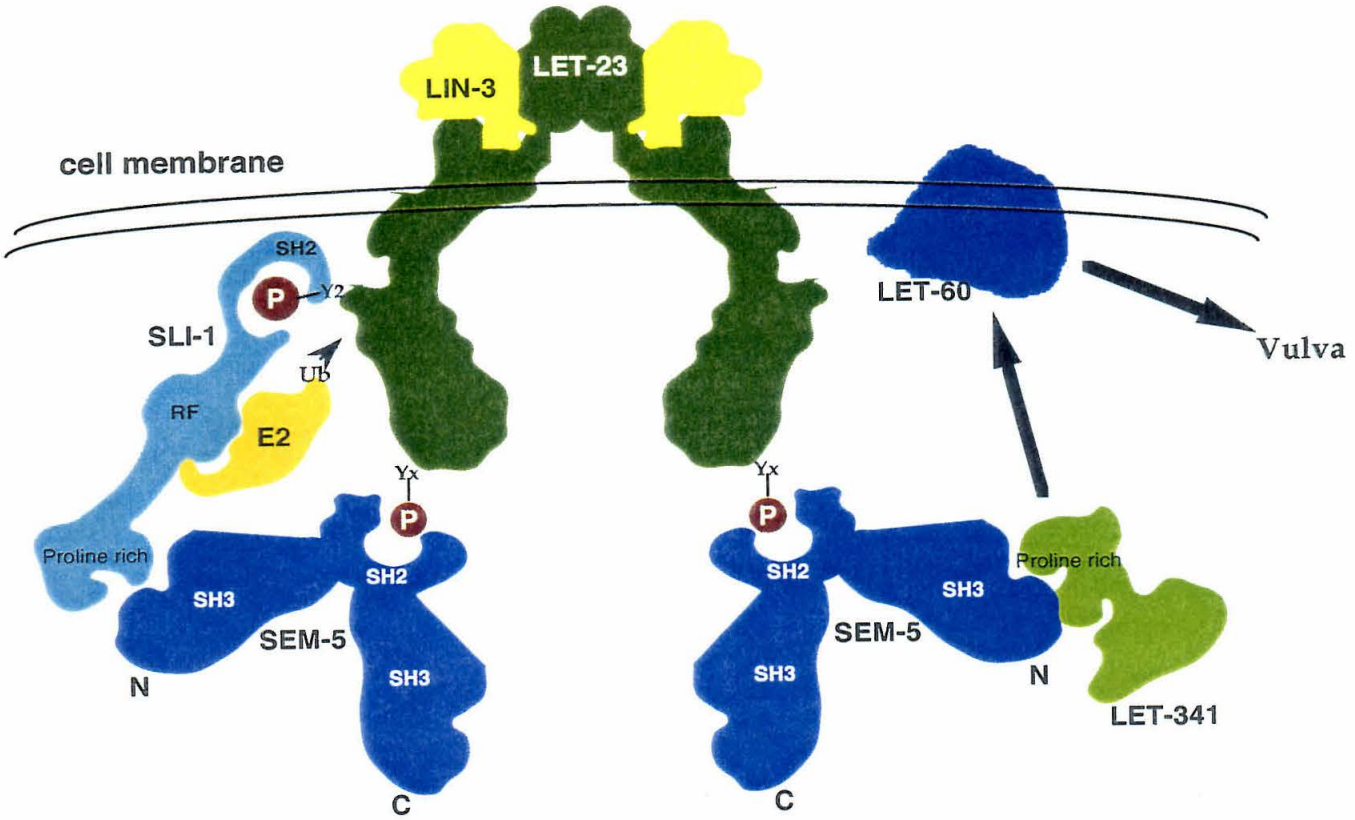

LIN-3
LET-23
cell membrane
SH2
P
Y2
SLI-1
Ub
RF
E2
Proline rich
SH3
SEM-5
SH2
SH3
N
C
Yx
P
LET-60
Vulva
Proline rich
LET-341

Figure 2.

Nomarski micrographs of vulval induction in various genetic backgrounds. (**a**) wild-type. (**b**) *sli-1(sy143)*. (**c**) *sem-5(n1619)*. (**d**) *sli-1(sy143) sem-5(n1619)*. (**e**) *sem-5(ay73)*. (**f**) *sli-1(sy143) sem-5(ay73)*. (**a**), (**b**) and (**d**) have phenotypically wild-type vulvae. (**c**), (**e**) and (**f**) are phenotypically Vul where all VPCs have generated epidermal progeny (note the lack of vulval openings in these panels). In all panels, anterior is to the left and dosal is towards the top. The scale bar is 20 μm.

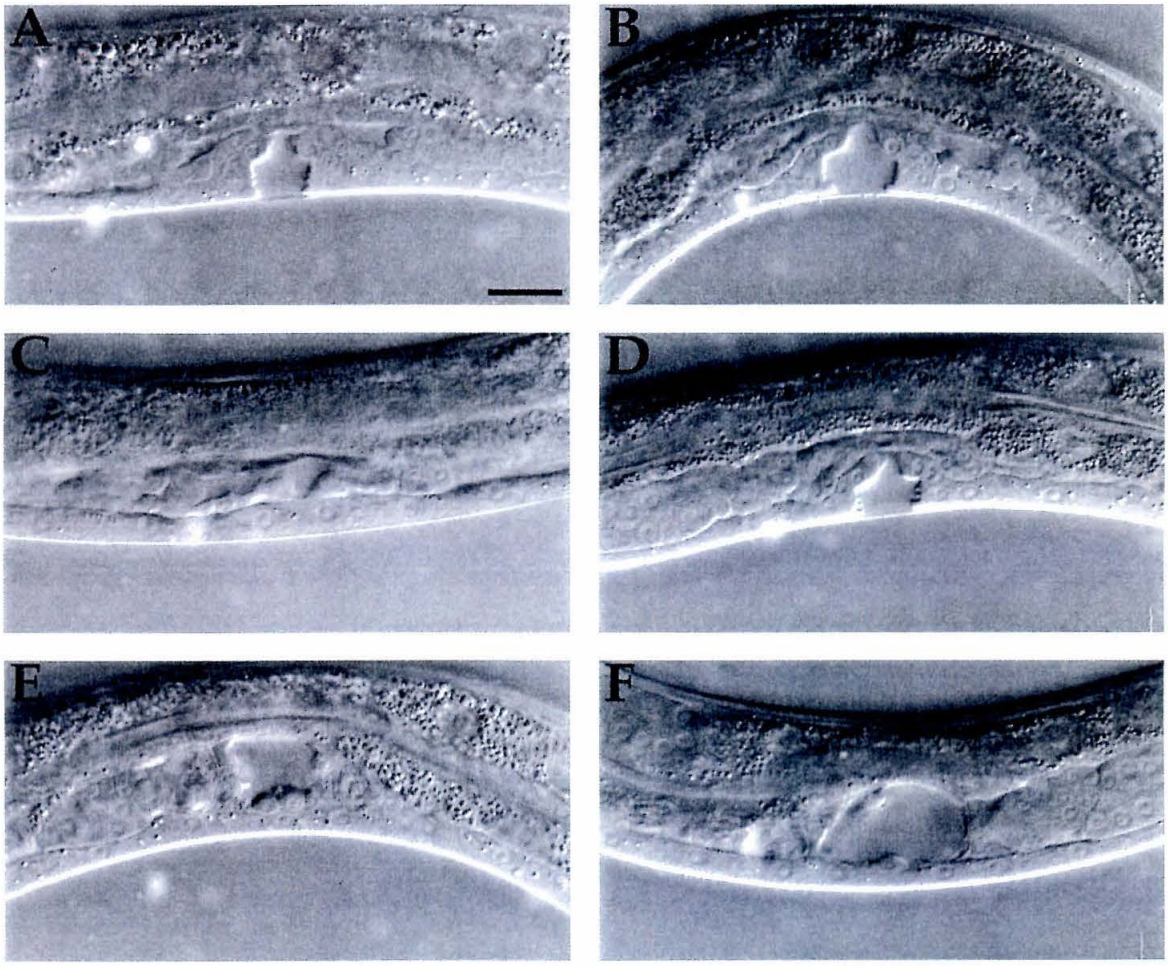

A
B
C
D
E
F

Figure 3.

Schematic alignment of SLI-1 and human c-Cbl. Conserved domains between the two proteins have been marked in the same manner. Residues 58 to 447 of SLI-1 share 55% amino acid sequence identity to residues 45 to 437 of human c-Cbl. The four helix bundle (4H), EF hand, SH2 domain, the RING finger, and the putative SH3-binding polyproline domains are indicated. Arrow marks the C-terminal truncation point of v-Cbl. The figure is a modified version from Yoon et al. 1995 and Meng, et al. 1999. The keys for this figure are retained in the structure-function analysis presentation in Figure 4.

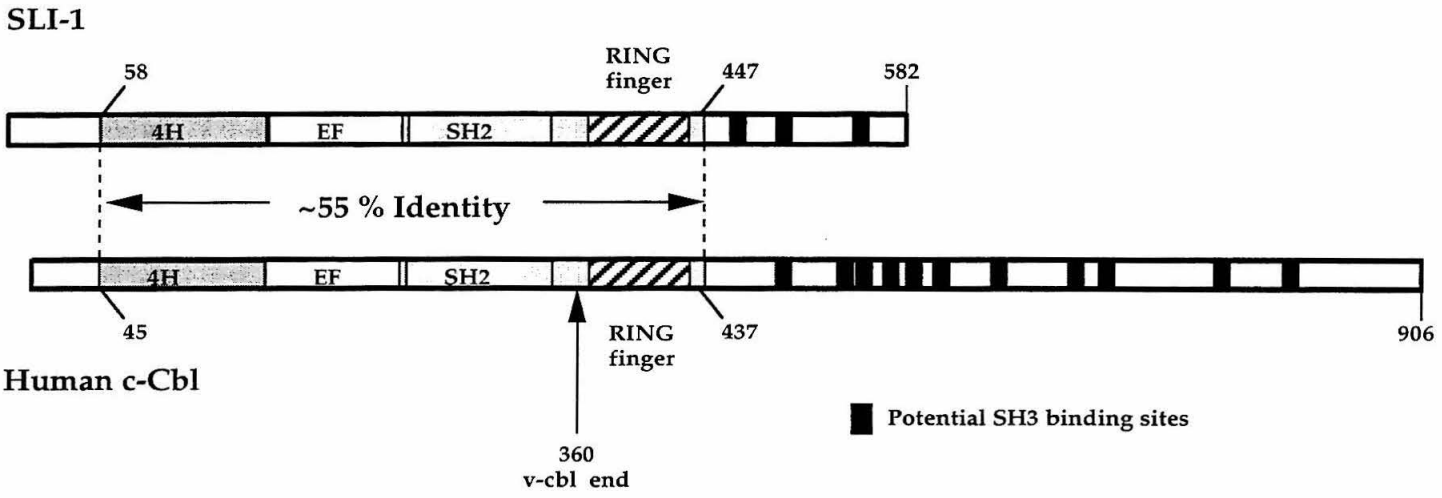
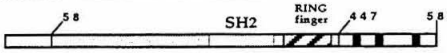
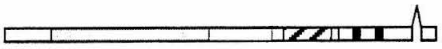
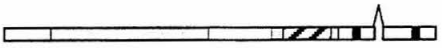
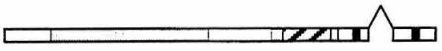
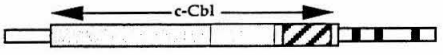

SLI-1
58
RING
finger
447
582
4H
EF
SH2
~55 % Identity
4H
EF
SH2
45
RING
finger
437
906
Human c-Cbl
Potential SH3 binding sites
360
v-cbl end

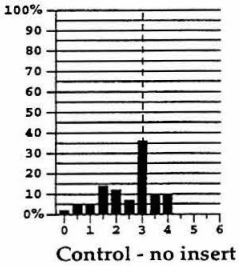
Figure 4.

(A) Vulval differentiation in germline transformed progeny carrying various minigene constructs, as indicated. Vulval differentiation is scored in L4 non-Dpy progeny following heat shock in early L3 (genotype *let-23(sy1); dpy-20; sli-1; syEx[heat shock construct + pMH86(dpy-20+)]*). The first column contains schematic representations of the mutant constructs inserted into the heat shock minigene pPD49.83. The second column lists construct designations. Average number of VPCs that undergo vulval differentiation are calculated in the third column for each independent stable line. The results are averaged from the combined data of column 3 in column 4. (B) Histograms. The distribution of vulval differentiation levels of the combined data (column 4, part A) obtained using the various heat shock minigene constructs described in part A. Horizontal scale (1-6) represents the number of VPCs that became vulval tissue per worm, scored in half-cell increments. Vertical scale (0% - 100%) represents the percent of total worms scored with a given level of vulval differentiation. The constructs are listed directly below each histogram and the names correspond to the construct designations of part A.

A

Injected sli-1 heatshock minigene constructs (schematic)	Construct designation	Average number of VPCs undergoing vulval differentiation (individual lines)	Average number of VPCs undergoing vulval differentiation (combined)
No cDNA insert	Control	2.54 (n=40) 2.42 (n=18)	2.5 (n=58)
	SLI-1	0.36 (n=40) 0.56 (n=40)	0.5 (n=80)
	SLI-1.ΔPro3	0.30 (n=33) 0.37 (n=30)	0.3 (n=63)
	SLI-1.ΔPro2	0.47 (n=31) 1.00 (n=24)	0.7 (n=55)
	SLI-1.ΔExon10	0.82 (n=26) 0.89 (n=30)	0.9 (n=56)
	SLI-1.ΔC	0.86 (n=40) 1.14 (n=35)	1.0 (n=75)
	SLI-1.ΔNΔRING	2.66 (n=40) 2.16 (n=40)	2.4 (n=80)
	SLI-1.Δmyr	0.48 (n=35) 0.55 (n=32)	0.5 (n=67)
	SLI-1.ΔCΔmyr	0.89 (n=53) 0.83 (n=45)	0.9 (n=98)
	SLI-1.ΔNΔRING Δmyr	2.46 (n=40) 2.90 (n=20)	2.6 (n=60)
	SLI-1.ΔNΔRING ΔC	2.56 (n=40) 2.40 (n=40)	2.5 (n=80)
	SLI-1.ΔNΔRING +CBL.N-RING	1.38 (n=60) 1.75 (n=40)	1.5 (n=100)
	SLI-1.ΔRING	1.19 (n=31) 1.69 (n=35)	1.5 (n=66)

B



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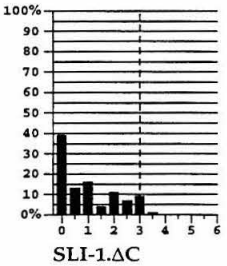
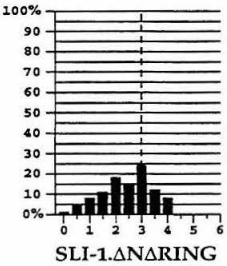


SLI-1.ΔC



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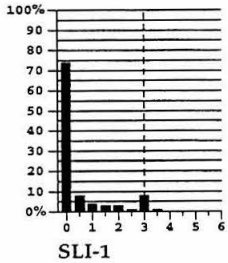
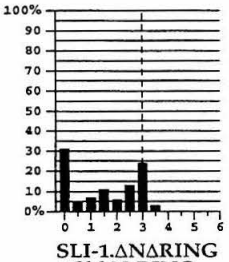
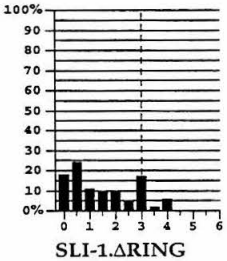


SLI-1



SLI-1.ΔNΔRING +Cbl.N-RING



SLI-1.ΔRING

Chapter 4

Receptor tyrosine phosphatase CLR-1 negatively regulates EGFR signaling

Recent studies in mice suggest EGFR functions as a survival factor in oncogenic transformation and provides a valuable target for therapeutic intervention. By comparative analysis of two RTK pathways with similar signaling mechanisms, we demonstrate that *clr-1*, a previously identified negative regulator for *egl-15* mediated FGFR signaling, is also involved in *let-23* EGFR signaling. Specifically, a reduction-of-function mutation in *clr-1* suppresses the lethality and vulvaless phenotypes of non-null mutations in *lin-3* EGF, *let-23* EGFR, *sem-5* Grb2, and *let-341* Sos and thereby rescuing their vulvaless and lethal phenotypes. Further, a *clr-1(rf)* mutation synergized with an activated *let-60* Ras mutant and with mutations in any other loci defining negative regulators of *let-23* EGFR mediated vulval signaling, including *sli-1*, *ark-1*, *unc-101*, and *gap-1*. Epistasis analysis orders *clr-1* action upstream of *let-60* Ras. Since *clr-1* encodes a single pass transmembrane protein with two putative tyrosine phosphatase domains in its carboxyl terminus, it is conceivable that CLR-1 acts to inhibit EGFR signaling by dephosphorylating active LET-23. Here, we show that RTKs share common mechanisms of negative regulation and that EGFR signaling as well as FGFR signaling is regulated by the Clr recognition of tyrosine kinase. This study presents a feasible approach to identifying new players in RTK pathways and we show that CLR-1 is the first receptor tyrosine phosphatase discovered in any model system acting to attenuate EGFR signaling.

Excessive or prolonged RTK signaling is associated with, and may be the cause of, both malignant transformation and progression of multiple tumour types [1, 2, 3]. Therefore, proper mechanisms must be employed to strongly control the signaling from RTKs to prevent spontaneous, ligand-independent activation and downregulate signaling after it has been activated. Negative regulators of EGFR signaling therefore have significant pharmaceutical potential, either as therapeutic agents themselves, or by promoting a strategy to engineer therapeutic molecules. There are three such inhibitors identified recently in *Drosophila* and four in *C. elegans*. For *Drosophila*, they are Argos, Kekkon-1, and Sprouty, which encode a secreted protein with an atypical EGF motif, a transmembrane adhesion molecule, and a novel Drk and RasGap1 interacting protein, respectively [4,5,6]. All three are transcriptionally dependent on EGFR signaling and participate in negative feedback control of receptor activation. This feature allows complex developmental patterning. For *C. elegans*, they are UNC-101, GAP-1, ARK-1, and SLI-1, encoding a homolog of mammalian AP-47 clathrin medium chain protein, a Ras-GTPase activating protein, a non-receptor tyrosine kinase related to Ack, and a Cbl proto-oncoprotein, respectively [7,8]. Mutations in each of these four negative regulators are silent by themselves. However, in combination with mutations in any other loci defining negative regulators results in a Multivulval (Muv) phenotype.

Generally, there are two ways to identify genes involved in the *let-23* EGFR-mediated vulval induction pathway. Genetic screens have been proven efficiently to uncover loci determining vulval cell fates. Direct screens for the Multivulva and the Vulvaless mutants reveals several major positive effectors of the pathway. These mutants were then served as the starting materials to look for the remaining members of positive effectors and negative regulators by extragenic suppressors screens. Sequence homology searches, motivated by new

components discovered in EGFR signaling pathway in other model organisms, also helped to identify several modulators in *C. elegans*, for example *mpk-1* and *sos-1*. The approach we adopted in this study is the comparative analysis of two RTK pathways with the similar signaling logic. We started with the alignment of FGFR pathway against EGFR pathway, candidate genes that modified *egl-15* FGFR were tested for their ability to alter signaling by the *let-23* EGFR (reasoning that the signal transduction pathways downstream of different RTKs would be similar). *clr-1*, a previously described inhibitor of *egl-15* FGFR signaling [9], is found to regulate *let-23* EGFR signaling activity in our vulval assay. The conceptual translation of *clr-1* transcript predicts a transmembrane domain followed by two intracellular tyrosine phosphatase domains [9]. The localization of CLR-1 at the periphery of body muscles is consistent with it acts as a receptor tyrosine phosphatase [9]. The temperature-sensitive mutation *clr-1(e1745ts)* was used in the following studies. This *clr-1* mutation was found to correspond to a nonsense mutation affecting the very carboxyl terminus of the D1 phosphatase domain (W1072-opal), resulting in the destabilization of CLR-1(e1745) protein at the nonpermissive temperature [9]. *clr-1* mutation suppresses vulvaless and lethal phenotypes caused by severe reduction-of-function mutations in positive effectors of *let-23* EGFR pathway (Table 1, 2). LET-23 is induced by LIN-3, an EGF-like growth factor and is properly localized to the basolateral membrane domain of vulval precursor cells (VPCs) by the PDZ protein LIN-10 [10,11]. LET-23 activation initiates a signaling cascade that involves downstream effectors such as SEM-5, LET-341, LET-60, LIN-45, MEK-2 and SUR-1/MPK-1 which are the nematode counterparts of the mammalian Grb2 adaptor, the Sos guanine nucleotide exchanger, the Ras GTP-binding protein, the Raf serine/threonine kinase, the MAPK kinase and the MAPK, respectively [7 12]. Animals with significantly reduced activities in *lin-3* EGF, *let-23* EGFR, *lin-10* PDZ, *sem-5* Grb2,

and *let-341* Sos display high penetrance of vulvaless phenotypes. Their vulvaless phenotypes were reversed by the reduced CLR-1 activity (Table 1). Rescue of vulval phenotype was determined based on two criteria, the average vulval induction and proportion of animals with less than three cells induced. The inference drawn from these observations is that CLR-1 normally acts as a negative regulator of EGFR signaling during vulval induction. Although mutation in *clr-1* suppresses strongly a severe reduction-of-function mutation in *sem-5*, it does not bypass the requirement of *sem-5* for EGFR-mediated vulval signaling since it does not overcome a *sem-5* null mutation. To address the question of whether the effect of *clr-1* on EGFR signaling is indirect through its regulation on *egl-15* FGFR, we introduced a severe *egl-15* mutation into the double mutant of *clr-1(e1745) let-23(sy1)* and test if the up-regulation of EGFR signaling by *clr-1* mutation is dependent upon *egl-15* activity. The elevated vulval signaling conferred by the *clr-1* mutation in a *let-23* EGFR mutant is not abolished by the *egl-15* mutation, suggesting that the effect of *clr-1* on EGFR signaling is most likely direct and not mediated through FGFR signaling. Although *clr-1(e1745ts)* is silent with regards to vulval induction by itself, in combination with a mutation in any other loci defining negative regulators results in a Muv phenotype (Table 1). Animals of the genotype *clr-1(e1745); gap-1(n1691)* have a hyperinduced vulva phenotype with 35% of animals having greater than three VPCs induced at 15°C (average number of VPCs induced = 3.2; Table 1). *clr-1* also synergizes with mutations in *ark-1*, *sli-1* and *unc-101*, resulting in a hyperinduced vulva phenotype. The percentile of animals having greater than three VPCs induced ranges from 15 to 40 (Table 1). Double mutants of *clr-1(e1745); gap-1(n1691)* can not be assayed at 20°C since animals are not viable. Interestingly, animals of the genotype *unc-101(sy108); clr-1(e1745)* display both vulvaless and Muv phenotype, suggesting that UNC-101 has both positive

and negative effects on the EGFR signaling. This feature is similar to that of proteins involved in localizing LET-23 to the sub-membrane domain of VPCs, indicating that UNC-101 might function in a similar way.

C. elegans development uses EGFR signaling repetitively; mediating vulval induction, viability, male spicule development, hermaphrodite ovulation, and differentiation of the ventral uterus and posterior ectoderm [7]. The alleles for *lin-3*, *let-23* and *lin-10* we used in this study do not cause any noticeable lethality, while *sem-5* and *let-341* display high penetrance of embryonic and early larval lethality. The lethality displayed by the reduction-of-function *sem-5* and *let-341* mutants is significantly suppressed by the *clr-1* mutation, further supporting an inhibitory role for *clr-1* in EGFR-mediated signaling (Table 2). While *clr-1(e1745ts); sem-5(n1619)* and *clr-1(e1745ts); let-341(ku231ts)* double mutants can be maintained at the nonpermissive temperature, the individual single mutants can not. The lethal phenotype of *clr-1* mutation is due to the excessive FGFR signaling while that of *sem-5/let-341* mutation is due to insufficient EGFR signaling. The mutual suppression between severe alleles of *clr-1* and *sem-5/let-341* observed here suggests that *clr-1* and *sem-5/let-341* are important for both EGFR and FGFR signaling. To determine the site of action for *clr-1*, we eliminated by gonad ablation the signaling flow upstream of an activated *let-60* Ras mutant and test the ability of *clr-1* to regulate the signaling flow elicited by the activated RAS variant; Ablation of gonad eliminates the ligand for *let-23* mediated vulval signaling [13]. The activated RAS allele, *n1046*, used in this study is still sensitive to the regulation by the ligand activation [12]. While *clr-1* mutation synergizes with *n1046* mutation in the presence of gonad, it does not in the absence of the gonad. Since *clr-1* mutation does not enhance the signaling flow from the activated RAS, we interpret that *clr-1* normally acts upstream of *let-60* Ras to regulate the EGFR signaling (Table 3).

In this study, we have demonstrated that *clr-1*, a previously identified negative regulator of *egl-15* mediated FGFR signaling, is also involved in *let-23* EGFR signaling. CLR-1 is the first receptor tyrosine phosphatase discovered in any model system acting to attenuate EGFR signaling. The success of this approach promises a similar reciprocal test and could potentially extend to the study of other signaling pathways with similar signaling logic. Indeed, our preliminary results suggest that one of the negative regulators of *let-23* EGFR-mediated signaling is also involved in *egl-15* FGFR signaling (our unpublished results) . The negative regulatory circuit operating on FGFR signaling in *C. elegans* is apparently dominated by *clr-1* since a *clr-1* mutation by itself expresses the clr phenotype of an activated *egl-15* FGFR variant. In contrast, the negative regulatory circuit operating on EGFR signaling in *C. elegans* is composed of multiple functionally partial redundant inhibitors. The complexity of this regulatory network in the vulva ensures a robust developmental consequence. Alternatively, it might provide the temporal and spatial specificity for EGFR signaling during the inductive event, e.g. EGFR signaling is reused a few hours later [14].

Methods

Strain construction and maintenance

Strains were maintained at 20°C and handled according to Brenner (1974). The following alleles were used for strain construction: for LGI, *unc-101(sy108)*, *lin-10(e1439)*; for LGII, *clr-1(e1745)*, *let-23(sy1)*; for LGIV, *lin-3(n378)*, *ark-1(sy247)*, *let-60(n1046)*; for LGV, *let-341(ku231)*, for LGX, *egl-15(n1477)*, *sli-1(sy143)*, *sem*

5(n1619, ay73) (Clark et al. 1992a; M. Stern, personal communication), *gap-1(n1691)*.

Vulval induction assay

The extent of vulval induction was determined by examining vulval anatomy in the early to mid-L4 stage of development under Nomarski optics using a Plan 100 objective [15]. Wild-type animals have three VPCs generating vulval progeny; vulvaless animals have fewer than three VPCs generating vulval progeny; multivulva or hyperinduced animals have more than three VPCs generating vulval progeny. In some cases, a VPC generates one daughter that makes vulval progeny and another daughter that becomes nonvulval epidermis; such VPCs are scored as one-half VPC differentiating into vulval tissue.

Cell ablation

Ablations of the AC was performed with a laser microbeam as described by Sulston and White (1980).

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Table 1 *clr-1* mutation restores reduction-of-function in *let-23* signaling and synergizes with mutations in other negative regulators

Genotype	Average number of VPCs induced		At temperature
	clr-1(+)	*clr-1(e1745)*	
+/+	3.0 (n=many)	3.0 (n=20)	20°C
lin-3(n378)	0.4 (n=25; %<3=96)*	2.0 (n=28; %<3=46)*	20°C
let-23(sy1)	0.8 (n=29; %<3=)#	2.0 (n=27; %<3=)*	20°C
lin-10(e1439)	0.5 (n=20; %<3=95)	2.7 (n=24; %<3=33)	20°C
sem-5(n1619)	0.4 (n=40; %<3=98)	2.7 (n=20; %<3=25)	20°C
sem-5(ay73)	0.0 (n=18; %<3=100)	0.0 (n=20; %<3=100)	20°C
let-341(ku231)	2.1 (n=20; %<3=60)	3.0 (n=20; %<3=20)	20°C
let-341(ku231)	n.d	0.9 (n=20; %<3=95)	25°C
gap-1(n1691)	3.0 (n=20; %>3=0)	3.2 (n=20; %>3=35)	15°C
ark-1(sy247)	3.0 (n=20; %>3=0)	3.3 (n=20; %>3=30)	15°C
ark-1(sy247)	3.0 (n=20; %>3=0)	3.4 (n=20; %>3=35)	20°C
sli-1(sy143)	3.0 (n=20; %>3=0)	3.4 (n=60; %>3=30)	20°C
unc-101(sy108)	3.0 (n=20; %>3=0)	2.9 (n=20; %<3=35; %>3=15)	20°C

*data of N. Moghal, personal communication.

#data from Jongeward et al. (1995).

Table 2 *clr-1* mutation suppresses lethality caused by the reduced *let-23* signaling

Genotype	% viability	At temperature
sem-5(n1619)	0 (n=many)	20°C
clr-1(e1745); sem-5(n1619)	40 (n=464)	20°C
let-341(ku231)	7 (n=135)	25°C
clr-1(e1745); let-341(ku231)	78 (n=128)	25°C

Table 3 Ordering site of action for *clr-1*

Genotype			Average vulval induction	Proportion of animals with
clr-1	*let-60*	*gonad*		>3 cells induced
1. +	*n1046*	+	3.5	10/20
2. *e1745*	*n1046*	+	4.2	18/20
3. +	*n1046*	-	0.7	0/9
4. *e1745*	*n1046*	-	0.8	0/8

Chapter 5

Reciprocal EGF signaling back to the uterus from the induced *C. elegans* vulva coordinates morphogenesis of epithelia

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Background: Reciprocal signaling between distinct tissues is a general feature of organogenesis. Despite the identification of developmental processes whose coordination requires reciprocal signaling, little is known regarding the underlying molecular details. Here, we use the development of uterine-vulval connection in *Caenorhabditis elegans* as a model system to study reciprocal signaling.

Results: In *C. elegans*, development of the uterine-vulval connection requires the specification of uterine uv1 cells and morphogenesis of 1°-derived vulval cells. LIN-3, an EGF family protein, is first produced by the gonadal anchor cell to induce vulval precursor cells to generate vulval tissue. We show that *lin-3* is also expressed in the 1°-vulval lineage after vulval induction. In addition, and that the 1° vulva is necessary to induce the uv1 cell fate. Using genetic and cell biological analyses, we find that the specification of uterine uv1 cells is dependent of EGF signaling from cells of the 1° vulval lineages to a subset of ventral uterine cells of the gonad. RAS and RAF are necessary for this signaling. We also find that EGL-38, a member of the PAX family of proteins, is necessary for transcription of *lin-3* in the vulva but not the anchor cell. A *let-23* mutation that confers ligand-independent activity bypasses the requirement for EGL-38 for specification of the uv1 cell fate.

Conclusions: We have shown how relatively simple EGF signals can be used reciprocally to specify the uterine-vulval connection during *C. elegans* development.

Background

During metazoan development, patterns of cell types are often established by inductive signaling between tissues. The molecular basis of inductive signaling can involve peptide signals (growth factors) acting through transmembrane receptors to activate signal transduction pathways in responding cells [1,2]. Reproducible pattern formation likely involves tight regulation of ligand production in the inductive cell and threshold-setting mechanisms in the target cell. LIN-3 is used multiple times as an inductive signal during development of *Caenorhabditis elegans*. The responses are likely mediated through LET-23, an EGF receptor tyrosine kinase. The outcome of signaling is context-dependent. Reduction-of-function mutations in both *lin-3* and *let-23* can affect the development of vulva, male spicules and posterior ectoderm, cause larval lethality and result in hermaphrodite sterility [3-7]. The question of how the production of LIN-3 is regulated in a tissue-specific manner is important because in a system in which multiple responses can occur in response to the relative strength of signaling, the precise response can be modulated by control of ligand production. The organization of *cis*-regulatory elements in *lin-3* genomic DNA and the transcription factors which bind to them will provide mechanisms which ensure precise spatial and temporal pattern of *lin-3* expression. A 5 kb *lin-3* genomic region that confers anchor cell (AC)-specific expression [8] has been identified.

There are several steps in the formation of the uterine –vulval connection. The first step is to induce vulval and uterine development by the same inductive source (AC), which ensures that the two share a center for the future registration [9]. The AC first induces three of six multipotential vulval precursor cells (VPCs) to adopt vulval cell fates via LIN-3/LET-23-mediated signaling [3,8,10-14] and 4

hours later induces six of twelve multipotential ventral uterine intermediate precursor cells to adopt the π cell fate via LAG-2/LIN-12-mediated signaling ([15]; A. Newman and P. Sternberg, unpublished observations). The inductive signaling pathways likely lead to changes in gene expression, such that specific vulval and uterine cell fates are executed, and ultimately that a functional uterine-vulval connection is ensured. The subsequent morphogenetic events include the following. The vulval cells form an invagination, with 1° vulval cells at the apex of the invagination (vulF) attached to the ventral most uterine cells (π cell progeny). All the π cell progeny (except the four which make the direct contact with vulF and become uv1 cells) fuse with the AC to form the thin laminar process (utse cells) that resides between the uterus and the vulva [16]. This fusion and differentiation of the utse moves the bulky AC and extra π cell progeny out of the way, thereby permitting a connection to be formed. In the meantime, the 1° vulval cells separate anterio-posteriorly and left-right to create a hole so that eggs can pass through it. Two sets of observations led us to consider that proper morphogenesis of the uterus might be dependent on signaling from the vulva to the uterus. We observed that *lin-3* is expressed in the vulva and *let-23* is expressed in the uterus; and that uv1 cells are absent in animals lacking vulval tissue. In addition, we examined existing mutants for the abnormal expression of *lin-3* and found a tissue-specific regulator for *lin-3*.

Results

The expression pattern of *lin-3* and *let-23* during the development of the connection between uterus and vulva

We constructed a *lin-3::lacZ* expression vector containing 12.5 kb *lin-3* genomic

sequence with approximately 2 kb regulatory sequence 5' to the transcriptional start site, which was inferred from known cDNAs ([8]; P. Tzou, R. Hill and P. Sternberg, unpublished observations). This construct is expressed in several tissues as expected from previous genetic analysis (data not shown). AC expression is required for vulval development (see [8]). Spermatheca expression might be involved in hermaphrodite fertility function. K lineage expression might be required for the development of posterior ectoderm. In all cases expression can be detected at the appropriate time. In addition, we found unexpected vulval expression from the early to mid fourth larval (L4) stage in the vulF cells, which are the dorsal most 1° vulval progeny (Figure 1D,E). The expression appears to be 1° vulva specific since the vulval *lin-3::lacZ* expression is absent in animals bearing a strong *lin-12(gf)* mutation (data not shown), which confers a multivulva phenotype consisting of only 2° lineages [17]. The presence of multiple copies of this construct induces a multivulva phenotype with low penetrance with all the vulval invaginations expressing *lin-3::lacZ* (Figure 1F). Since the uterus-connected 1° vulva is not the only one whose cells express *lin-3::lacZ*, it is unlikely that a second gonad dependent signal is required late to induce *lin-3::lacZ* expression in the 1° vulva derived cells.

To determine whether this expression could have functional significance, we examined the expression pattern of *let-23::GFP* during the period which corresponds to vulval expression of *lin-3::lacZ*. A S65T GFP cassette was fused in frame in an intact *let-23* genomic clone, pk7-13.8, which rescues the defects associated with loss-of-function alleles of *let-23* [11]. We detected expression of *let-23::GFP* in the uv1 cells, which are the ventral most uterine cells, suggesting possible signaling from the vulva to the uterus (Figure 1B,C). Since expression of *let-23::GFP* in uv1 cells peaks later than the proposed induction, its expression might be upregulated by *lin-3*. We have not formally demonstrated that *let-23* is

expressed more broadly than in the presumptive uv1 cells. Therefore, it is conceivable that the LIN-3/LET-23 induction is permissive.

Progeny of the 1° vulval cell lineage signal the uterus to specify the uv1 cell fate

In vulvaless *let-23(sy97)* hermaphrodites, in which all six VPCs adopt the non-vulval 3° cell fate, the presumptive uv1 cells instead adopt a utse cell fate (Table 1). Since the presumptive uv1 cells are born after the vulva is induced, these observations suggest that the vulva might be necessary for the uv1 cell fate. To test this hypothesis, we used laser microsurgery to ablate all or some of the six VPCs. Ablation of either all 6 VPCs or of only P6.p (the progeny derived from P6.p adopt the 1° vulval cell fate in a wild-type animal) results in transformation of the uv1 cell fate into a utse cell fate (Table 1). Ablation of all the VPCs except P6.p does not affect uv1 cell fate. These observations suggest that uv1 specification is dependent upon a signal from the 1° vulval cell lineages. 2° vulval cell lineages (descendants of P5.p and P7.p) appear to be neither necessary nor sufficient to induce the uv1 fate. To further localize the source of inductive signal, we ablated P6.p descendants at the 2 and 4 cell stage. In addition, we ablated just the F or E grandprogeny of P6.p (F, P6.pap and P6.ppa; E, P6.paa and P6.ppp) at the 4 cell stage. Our results suggest that the inductive signal is produced by P6.p descendants during or after the 4 cell stage. Ablation of the two E does not affect the uv1 cell fate, and ablation of two F has less effect than ablation of all 4 cells (E and F; uv1-to-utse transformation of 25% and 75% respectively). This observation suggests that either there is a window within the 4 cell stage used for the production of inductive signal in F lineages, or that E lineages can replace F lineages for the production of inductive signal when F lineages are removed.

LET-23-mediated signaling is required for uv1 cell specification

As discussed above, while vulvaless *let-23* hermaphrodites fail to specify the uv1 cell fate, this result alone is not sufficient to implicate the LIN-3/LET-23 pathway in vulva-to-uv1 signaling, since laser ablation of the VPCs also results in uv1-defective animals. We therefore examined animals containing partial loss-of-function mutations in genes functioning in the LIN-3/LET-23 signal transduction pathway mediating vulval induction. These strains had defects in uv1 cell fate specification (Table 2), indicating that multiple components of the vulval induction pathway are used again in vulva-to-uv1 signaling. In *let-60(n2021)* animals, the defect was only partially penetrant, consistent with the fact that the mutants studied can not be too severe as a functional vulva has to be specified in order to assay a more direct effect on uv1 specification. Mutations in other genes (*let-23*, *lin-45* and *lin-1*) result in a highly penetrant failure to specify the uv1 cell fate without severely affecting vulval induction, suggesting that these mutations preferentially affect one tissue, and that the defect in vulval induction is not responsible for the uv1 defect.

Another way to examine LET-23 pathway function in uv1 specification is to alleviate the vulval induction defect by mutating negative regulators. SLI-1, a homolog of the proto-oncogene product c-Cbl, is a negative regulator of LET-23-mediated vulval differentiation in *C. elegans*. [18,19]. GAP-1 is a negative regulator of vulval signaling that likely acts at the level of LET-60 Ras, similar to the Ras GTPase-activating proteins identified in other systems [20]. While mutations in SLI-1 and GAP-1 restore most of the reduced LET-23 signaling in the vulva caused by the severe hypomorph of *let-23*, with average vulval inductions of 2.9 and 2.7 respectively (Table 2), the specification of uv1 cell fate is

still compromised (Figure 2C,D). The high penetrance of uv1 defect observed in *let-23; gap-1* suggests that LET-23-mediated signaling is less regulated by GAP-1 in the presumptive uv1 cells than in the VPCs. However, since a weak hypomorph of *let-60* shows the uv1 defect, we believe that uv1 specification is a RAS-dependent event (see below).

RAS and RAF activities are required for the uv1 cell fate during the development of uterine-vulval connection

A *let-60::lacZ* reporter construct is expressed in the uv1 cells during the development of uterine-vulval connection ([21]; C. Chang and P. Sternberg, unpublished observations), and thus RAS might have a function in these cells at this time. The use of an inducible and tissue-general *hsp 16-41* heat shock promoter-driven putative dominant negative RAS variant (*hsrasDN*) allows us to test this possibility. This construct, which contains a G10R substitution in the *let-60* equivalent, is from another nematode, *Pristionchus pacificus* [22]. *C. elegans* hermaphrodites heterozygous for this G10R mutation have a reduction of gene activity and a vulvaless mutant phenotype [23]. Animals transformed with this construct, when subjected to heat shock after VPC cells have divided one round, had a low penetrance (7/33) vulval morphogenetic defect. Of animals that have a wild-type vulval morphology in the mid-late L4 stage (n=26), 50% had a failure to specify the uv1 fate (Table 3). Animals were judged to have a wild-type vulval morphology by three criteria: wild-type number of vulval cells; normal cell lineages; characteristic physical appearance. Most of these animals can lay eggs well in the adult stage, indicating wild-type function of their vulvae (see discussion).

lin-45(sy96) alters the 3′ splice acceptor of intron 4 of *Ce-raf*, resulting in

decreased vulval induction and viability [24]. Not all *lin-45* animals with wild-type extent of vulval induction observed at the mid-late L4 stage had wild-type vulval morphology. To avoid any complication of incompletely induced vulvae, we only scored animals with not only a wild-type extent of vulval induction, but whose vulval morphology was normal by four criteria: wild-type number of vulval cells, normal cell lineages, characteristic physical appearance and correct marker gene expression. Of these animals having a normal vulval morphology, there is a 92% failure to specify the uv1 fate (n=20) (Table 3). Despite this uterine defect, most of the animals can lay eggs well in the adult stage (17/20), further demonstrating the proper functioning of their vulvae (see discussion).

egl-38 acts upstream of _lin-3_ to regulate uterine differentiation

egl-38 is a member of the PAX family of genes, which encode transcription factors implicated in a variety of developmental patterning events. *egl-38(n578)* has defects in the egg-laying system resulting from the absence of uv1 cells and abnormal morphogenesis of 1° vulval tissue [25]. At the mid-L4 stage in wild-type animals, vulE and vulF generated by 1° vulval lineage, separate to produce a hole through which sperm and fertilized eggs pass. In *egl-38* animals, there is no defect in the number of vulval cell lineages produced, but vulF cells fail to separate (Figure 3A). The *egl-38(n578)* allele is a substitution of Glu for Gly 69 in the DNA-binding paired domain of EGL-38 [25]. We examined the expression pattern of *lin-3::lacZ* construct in the genetic background of *egl-38(n578)* and found that there is no expression of *lin-3::lacZ* in the vulva (Figure 3B); by contrast *lin-3::lacZ* expression in other tissues was unaffected. We infer that EGL-38 acts upstream of *lin-3* as a positive regulator of its transcription. Furthermore, the resulting lack of vulval *lin-3* expression in *egl-38* animals might be the cause

of the uv1 defect, the vulval morphogenetic defect involving a failure of the vulF cells to separate, or both.

let-23(sa62), a gain-of-function mutation of *let-23*, is able to suppress the uv1 defect in *egl-38* (Table 4). Thus, *let-23(sa62)* bypasses the requirement for *lin-3* expression in the vulva to mediate uv1 specification. While the uv1 defect is rescued by *let-23(sa62)*, the vulval morphogenetic defect remains. Therefore, the uv1 defect can not be the cause of the *egl-38* vulval morphogenetic defect. These experiments leave open the possibility that the vulval morphogenetic defect is the cause of uv1 defect, however, data from another mutant suggests that it is not (see discussion).

Discussion

We have shown that a vulval signal is needed to specify uterine π cell daughters as uv1 rather than utse. Using phenotypic analysis of mutants and *lin-3::lacZ* expression analysis, we have demonstrated that this signal is mediated by the same set of proteins - LIN-3, LET-23, LET-60, LIN-45 and LIN-1 - required for vulval induction.

A vulval signal is needed for uterine π cell daughters to adopt a uv1, rather than a utse, cell fate

During larval development of the *C. elegans* hermaphrodite, two sequential inductions by the AC specify the differentiated cells needed to connect the uterus and vulva, and thereby create a functional egg-laying system. First, the uterine AC induces a subset of the underlying epidermal precursor cells to adopt vulval cell fates and produce the vulva. The AC then induces adjacent ventral uterine

intermediate precursor cells to adopt the π (rather than the default ρ) cell fate and produce the two uterine cell types that connect to the vulva: utse and uv1. AC-to-π cell signaling requires the *lin-12*-encoded receptor. *lin-12* gain-of-function mutants [*lin-12(d)*], have a ρ-to-π cell fate transformation, observable under Nomarski optics, and a corresponding excess of utse tissue (EM analysis of one *lin-12(d)* mutant animal revealed 33 nuclei in the utse, compared to nine in the wild-type; [15]). EM analysis of *lin-12(d)* mutants did not reveal excess uv1 cells (John White, personal communication), suggesting that utse might be the ground state for π daughters, with an additional signal required for uv1. This hypothesis was supported by the finding that in *egl-38* mutants, presumptive uv1 cells instead adopt a utse cell fate [25]. Here we demonstrate that a signal from the vulF cells induces the directly overlying π cell daughters to become uv1; π daughters that do not receive this signal become utse. We also find that *lin-3* is expressed in the vulF cells at the right time and place to be the ligand for this induction; furthermore, specification of the uv1 cell fate is defective in animals bearing mutations in *let-23* or in downstream components of the LET-23-mediated vulval induction pathway, suggesting that these genes are required for uv1 cell fate specification as well.

Reciprocal EGF signaling from the vulva specifies the uv1 cell fate

The fact that *egl-38* has defects in the egg-laying system resulting from the absence of uv1 cells and abnormal 1° vulval morphogenesis, and the observation that vulval expression of *lin-3* specifically disappears in this genetic background, suggest that the expression of *lin-3* in the vulva might be required for uv1 specification, vulval morphogenesis, or both. Strong evidence that a 1° vulva-produced LIN-3 signal is required for uv1 specification comes from the

suppression of the uv1 defect in *egl-38* animals by the *let-23(sa62)* mutation. All known effects of LIN-3 are mediated through LET-23 and there is no evidence that LET-23 has additional ligands apart from LIN-3.

The failure of the *let-23(sa62)* mutation to suppress the vulval morphogenetic defect of *egl-38* animals raises the possibility that this defect is not mediated through LIN-3 expression. However, the dosage of *let-23(sa62)* might not be strong enough to suppress vulval morphogenetic defect caused by the *egl-38* mutation or the *let-23(sa62)* mutation might have cell-type specificity. Moreover, in addition to LIN-3, there could be another signal, also regulated by EGL-38, required for vulval morphogenesis.

A *lin-3::lacZ* transgene is expressed in the 1°-derived vulval cells in the early L4 stage, as well in the AC as reported previously [8]. Since the AC and the 1°-derived vulval cells are in proximity to the presumptive uterine-vulval connection, LIN-3 produced by either cell could be a potential inductive source required for the development of the uterine-vulval connection in the early-mid L4 stage. Ablation of P6.p descendants but not the AC after VPC cells have divided two rounds results in a uv1-to-utse cell fate transformation (Table 1). We infer that the inductive source used for uv1 cell specification is from the 1°-derived vulval cells and that LIN-3 expression in the AC is neither necessary nor sufficient to induce the uv1 cell fate. Consistent with this hypothesis, we observe *lin-3::lacZ* expression in the 1° vulva throughout the period when the uterine-vulval connection is developing, whereas *lin-3::lacZ* expression in the AC gradually disappears during this time interval (data not shown). We could not use the same experiments to address the issue of whether LIN-3 protein in the AC or 1° vulval tissue is used for the 1° vulval morphogenesis as suggested by the observations from *egl-38* mutants because physical invasion of the AC between the inner-most grand-daughters of P6.p, F lineages, is required for later

separation of the 1° vulval cells (K. Tietze and P. Sternberg, unpublished observations); and, P6.p descendants are the target we need to assay.

lin-3 regulation by EGL-38

Our finding that EGL-38 acts upstream of *lin-3* as a positive regulator suggests three possible models. First, EGL-38 might act upstream of gene *X*, which is required for vulval morphogenesis. Proper vulval morphogenesis would then allow *lin-3* to be expressed in the vulva to regulate uv1 specification. Second, EGL-38 might act upstream of *lin-3* to regulate uv1 specification and upstream of gene *X* to regulate vulval morphogenesis. These two events do not have an epistatic relationship. Third, EGL-38 might act upstream of *lin-3* to regulate uv1 specification and vulval morphogenesis. In addition to being necessary for *lin-3* expression in vulF cells, EGL-38 also regulates expression of other genes in the same cells (M. Wang, C. Chang and P. Sternberg, unpublished observations). We speculate that *lin-3* is an immediate target gene of EGL-38 in these cells.

The relationship between vulval morphology and uv1 cell fate

Even some mutants that confer a severely abnormal vulval morphology in both 1° and 2°-derived vulval cells do not affect vulval expression of *lin-3* and uv1 cell specification (unpublished observations). Proper vulval morphology is hence not required for vulval expression of *lin-3* and uv1 cell specification. By contrast, there are many examples of LET-23 signaling pathway mutants in which uv1 cell fate is compromised but vulval morphogenesis is normal. We infer that uv1 cell fate is not required for vulval morphogenesis. Therefore, uv1 cell specification

and vulval morphogenesis are independent events once a 1° vulval fate has been specified.

The working model

We favor the following model (Figure 4). EGL-38 acts upstream of *lin-3* as a positive regulator of its expression in the 1° vulval lineage. The 1° vulval expression of *lin-3* would then specify uv1 cell fate through RAS-RAF dependent signaling in a cell non-autonomous manner. *lin-3* produced by the 1° vulva could conceivably specify 1° vulval morphogenesis in a 1°-lineage autonomous manner. However, since the uv1 defect in an *egl-38* mutant is rescued by *let-23(sa62)* while the vulval morphogenetic defect remains, EGL-38 likely controls 1° vulval morphogenesis independent of its effect on *lin-3* expression.

Reciprocal signaling and organogenesis

Reciprocal signaling between distinct tissues is a general feature of organogenesis [26,27]. For example, during mouse nephrogenesis, several checkpoints occur at which a positive signal is needed for development to continue [28]. Reciprocal signaling is one mechanism for developmental checkpoints. In *Drosophila*, reciprocal signaling occurs between the approaching muscles and the epidermal muscle attachment cells during embryonic development [29]. The epidermal muscle attachment cells signal the myotubes and induce myotube attraction and adhesion to their target cells. Following this binding, the muscle cells signal back to the epidermal muscle attachment cells inducing their terminal differentiation into tendon-like cells.

In the *Drosophila* egg, an EGF signal (Gurken) from the oocyte patterns the

overlying follicle cells by inducing a dorsal-anterior follicle cell fate [30]. These specialized follicle cells then express another EGFR ligand, Vein and an accessory protein, Rhomboid, which are then used in an autocrine manner to amplify EGFR signaling in the follicle cells. Consequent high-level EGFR activation leads to localized expression of the diffusible inhibitor Argos, which modifies the initial EGFR activation profile, producing two peaks of activity displaced from the midline and hence the positions of the two dorsal appendages. In *C. elegans*, the initial paracrine signaling event by LIN-3 from the somatic gonad specifies vulval cell fates. We propose that this signaling event triggers expression of LIN-3 in the vulval cells, which in turn specifies the uv1 cell fate involved in the intimate connection of vulva and uterus. It is possible that LIN-3 expression in the vulva also has an autocrine amplification role upon which proper vulval development depends.

Conclusions

We find that the reciprocal EGF signaling from the vulva to the uterus coordinates the uterine-vulval connection. We demonstrate that uv1 cell specification is mediated by a RAS-RAF-dependent signaling transduction pathway. We show that *lin-3* expression in the 1° vulval cells, which is positively regulated by EGL-38, is required for uv1 cell specification. Since the uv1 defect but not the vulval morphogenetic defect of an *egl-38* mutant is rescued by activation of *let-23*, we think it likely that another signaling pathway, also regulated by EGL-38, is required for vulval morphogenesis in addition to or independent of LIN-3/LET-23. In general, since EGF Receptor activation of RAS is essential for the specification and differentiation of epidermal cells, the reciprocal EGF signaling might have evolved to mediate the coordinated

morphogenesis of epithelia as described here for the development of uterine-vulval connection.

Materials and methods

General methods and strains

C. elegans strain N2 and derivatives were maintained at 18°C-21°C and handled as described [31]. The following alleles were used: LGI, *ayIs4[egl-17::GFP; dpy-20(+)]* [32]; LGII, *let-23(sy97)* [4], *let-23(n1045)* [3], *let-23(sa62)* [33], *unc-4(e120)*; LGIV, *let-60(n2021)* [34], *lin-45(sy96)* [24], *lin-1(n1790)* [35], *egl-38(n578)* [36], *dpy-20(e1282)*; LGV, *him-5(e1490)* [37]; LGX, *sli-1(sy143)* [18], *gap-1(n1691)* [20], *unc-2(e55)* [31].

Nomarski microscopy and cell ablation

Vulval differentiation: The extent of vulval differentiation was measured as described previously [38]. The anatomy of L4 hermaphrodites were examined under Nomarski optics and the fate of individual VPCs inferred from the anatomy. Wild-type animals have three VPCs generating vulval progeny; vulvaless animals have fewer than three VPCs generating vulval progeny; multivulva or hyperinduced animals have more than three VPCs generating vulval progeny. In some cases, a VPC generates one daughter that makes vulval progeny and another daughter that becomes nonvulval epidermis; such VPCs are scored as one-half VPC differentiating into vulval tissue.

Uterine uv1 differentiation: By Nomarski optics, the π progeny that contribute to uv1 cells can be easily distinguished from those that become the utse since the nuclei of the latter undergo long-range migrations whereas those

of the former remain closely associated with the vulva. In wild-type animals, the nuclei of both ventral outer π progeny (VT4 and VT8) remain proximal to the vulva, while other π progeny nuclei (VT5, VT6, VT7, and VT9) co-migrate with the AC nucleus to either anterior or posterior of the presumptive vulval opening. For *let-23(sy97)* mutant animals and wild-type animals with P6.p daughters ablated at the two cell stage, π progeny nuclei were followed on either the left or the right side of the animal during mid to late L4 stage as described previously [16]. In these experiments, we observed a uv1-to-utse cell fate transformation, with VT4 and VT8 co-migrating with other nuclei of the utse. In the *let-23(sy97)* experiments, we sometimes observed a general delay in migration of π cell progeny and the AC; this may reflect the fact that in vulvaless animals, π daughters are born more ventrally, and contact the forming uterine lumen later, than in wild-type animals. In subsequent experiments, we scored the number of vulva-proximal cells with the characteristic uv1 cell morphology as our assay for uv1 cell fate specification.

Cell Ablations were performed with a laser microbeam as described previously [39].

Plasmids

The plasmid pCC2, containing the *lin-3::lacZ* fusion, was constructed as follows: A StuI-BamHI fragment containing 7.6 kb of a 5′ *lin-3* genomic sequence with 2 kb of regulatory sequence 5′ to the transcriptional start site was excised from the cosmid F36H1 and end-filled at the StuI site. A BamHI-SacI fragment containing the last 5 kb of a 3′ *lin-3* genomic sequence in pMob KS with a *lacZ* cassette fused in the first cytoplasmic exon of *lin-3* was excised from the plasmid pRH56 [8] and end-filled at the SacI site. The *lacZ* cassette includes a *trpS::lacZ* fusion, a nuclear

localization signal, and 3′ untranslated sequence from *unc-54*. These two fragments were ligated to create plasmid pCC2.

The plasmid pk7GL44.2, containing the *let-23::GFP* fusion, was constructed as follows: Site-directed mutagenesis of *let-23* genomic DNA was carried out, as described previously [40], in pk7-5.5, a HindIII clone of *let-23* that contains the last 3 kb of a 3′ coding sequence plus ~2 kb of 3′ untranslated sequence, so that the *let-23* stop codon and surrounding sequences were changed to an SphI site. At this site, a S65T *GFP* cassette [41] amplified by PCR with primers containing SphI site was cloned. This fragment was then excised with SalI and HindIII and ligated with an ~12-kb EcoRI-SalI genomic *let-23* fragment and with an 3-kb EcoRI-HindIII pBluescript II fragment generating the plasmid pk7GL44.2.

Construction of transgenic strains

Transgenic animals were generated by standard methods, which produce high copy number extrachromosomal arrays, commonly lost at mitosis and meiosis but still heritable [42]. Three sets of transgenes were used. The transgene *syEx241* was used to determine the expression pattern of the *lin-3::lacZ* fusion construct. It was obtained by microinjection of pCC2 at 27 ng/μl, pMH86 (*dpy-20(+)*) at 26 ng/μl, and carrier DNA (pSK+) at 100 ng/μl, into *dpy-20(e1282) egl-38(n578)* mutant animals, followed by crossing into *dpy-20(e1282)* to remove *egl-38(n578)* mutation but retain the extrachromosomal array. The transgene *syEx234*, used to analyze *let-23::GFP* expression, was obtained by microinjection of pk7GL44.2 at 100 ng/μl, pMH86 at 15 ng/μl, and carrier DNA at 60 ng/ul into *dpy-20(e1282)* mutant animals. The transgene *syEx284*, provided by M. Wang, was generated by injecting a solution of *hsrasDN* (pPD49.83:ras"149"; 10ng/μl), pMH86 (15 ng/μl) and carrier DNA (125 ng/μl) into *dpy-20(e1282)* mutant animals. This

transgene was used for the heat shock induced expression of dominant negative RAS variant. Heritable lines bearing the marker DNA (scored by rescue of *dpy-20(e1282)*) were tested for their ability to interfere with vulval and uterine differentiation in response to heat shock.

Analysis of lin-3::lacZ and let-23::GFP expression

X-gal staining was performed as described [43] except that acetone fixation was omitted as this better preserved the morphology of the animals. For characterization of GFP expression, fluorescence was observed on a Zeiss Axioplan microscope with a 200-watt HBO UV source, using a Chroma High Q GFP LP filter set.

Heat shock of transgenic animals

For heat shock of *syEx284*, worms in which the VPC cells had divided were placed on 5 cm culture plates seeded with bacteria, sealed in parafilm, and incubated in a covered water bath at 33°C 30-35 min.

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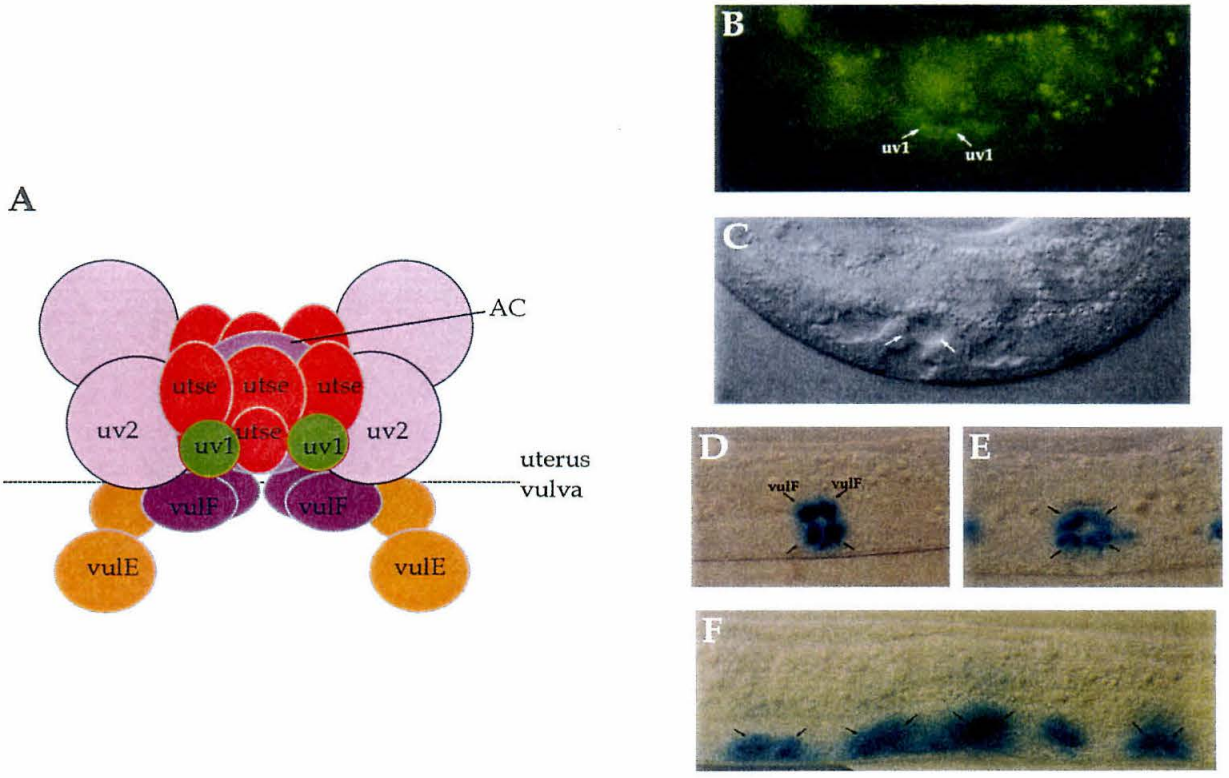
Figure 1. Expression pattern of *lin-3* and *let-23* reporter constructs in hermaphrodites during development of the uterine-vulval connection.
(A) Schematic representation of cell positions in the early phase of the developing uterine-vulval connection.
(B,C) GFP and Nomarski lateral images of a *let-23::GFP* transgenic hermaphrodite in the mid-L4 stage showing *let-23::GFP* expression in the uv1 cells (arrows) as a thin lateral ring connecting the uterus to the vulva. *let-23::GFP* was expressed from the *let-23* promoter in the *syEx234* transgenic array in a wild-type animal.
(D) Ventral view of a mid-L4 larva showing expression of β-galactosidase from the *lin-3::lacZ* reporter construct in the vulF descendants of P6.p (arrows).
(E) Ventral-lateral view of a mid-L4 larva showing *lin-3::lacZ* expression in the vulF cells (arrows).
(F) Lateral view of a mid-L4 larva with a multivulval phenotype showing *lin-3::lacZ* expression in all the vulval invaginations (arrows).


A
AC
utse
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uv2
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B
uv1
uv1
C
D
vulF
vulF
E
F

Figure 2. Nomarski photomicrographs illustrating the cellular defects in the uterine-vulval connections of mid-L4 *let-23; sli-1* mutants.
In wild-type (A) and *sli-1(sy143)* mutant (B) animals, the two specified uv1 cells (arrows) are proximal to the vulva. In *let-23(sy97); sli-1(sy143)* mutant animals (C,D) uv1 cell fate specification is abnormal. The anterior presumptive uv1 cell was not specified and has migrated dorsally rather than attaching to the vulva. In all panels, anterior is to the left and dorsal is toward the top. The nuclei of the vulE and vulF cells are also indicated.

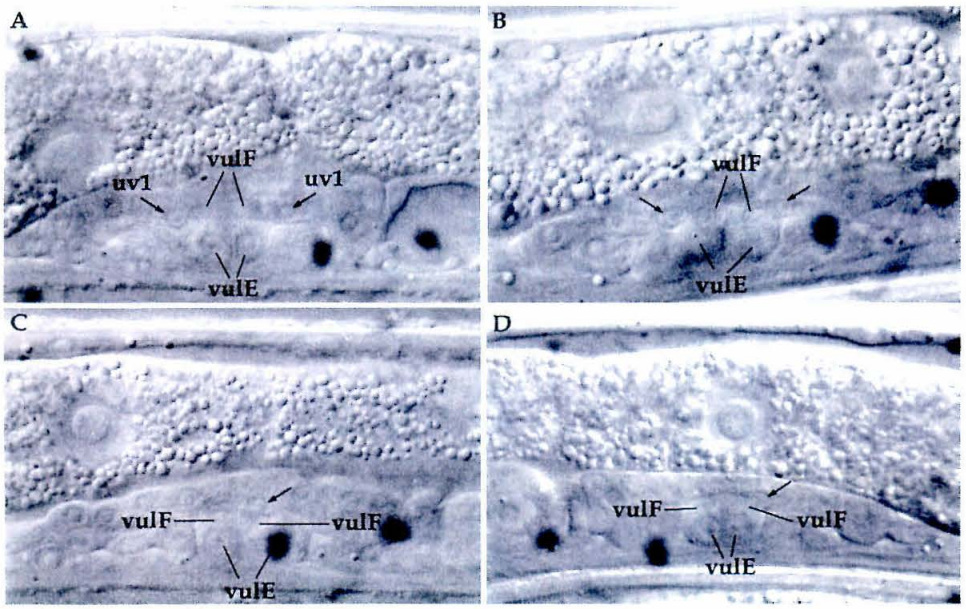

A
uv1
vulF
uv1
vulE
B
vulF
vulE
C
vulF
vulF
vulE
D
vulF
vulF
vulE

Figure 3. Expression of a *lin-3::lacZ* reporter construct is abolished in the vulF cells of *egl-38* animal.

(A) Nomarski photomicrograph of a mid-L4 *egl-38(n578)* hermaphrodite showing the failure of 1° vulval cells to separate.

(B) β-galactosidase expression can not be detected from the *lin-3::lacZ* reporter construct in the vulF descendants (arrows) of P6.p of a mid-L4 stage *egl-38* animal. Staining directly above the vulF cells represents expression in the partially out-of-focus anchor cell.

(C,D) Heterozygous *egl-38* animals were obtained by directly mating the *egl-38* transgenic hermaphrodites which carry the *lin-3::lacZ* reporter construct with N2 males. F1 cross progeny express β-galactosidase from the *lin-3::lacZ* reporter construct in the vulF descendants (arrows) whereas their homozygous parents did not.

Lateral (A-C) and ventral (D) views.

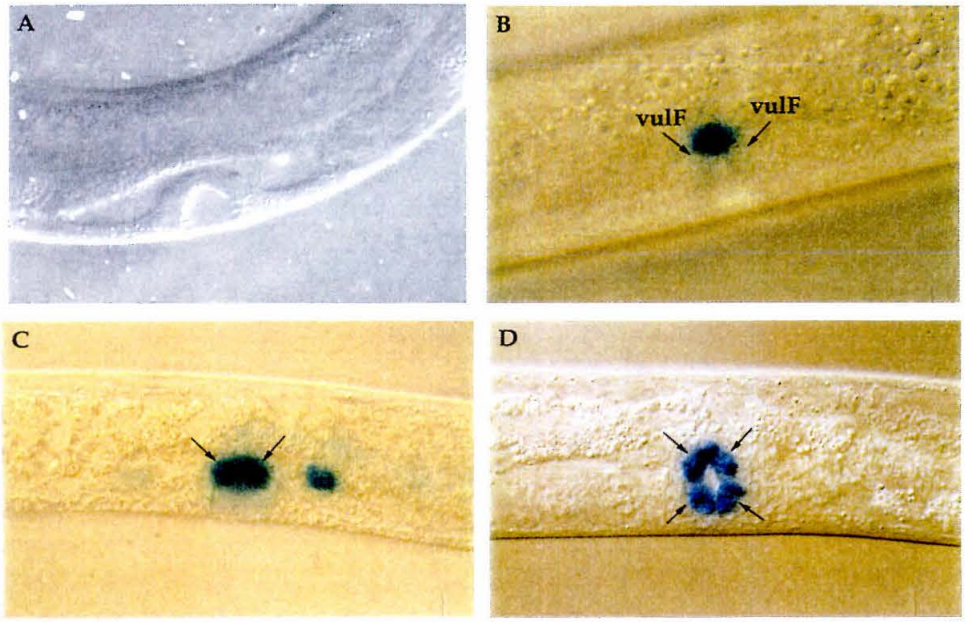
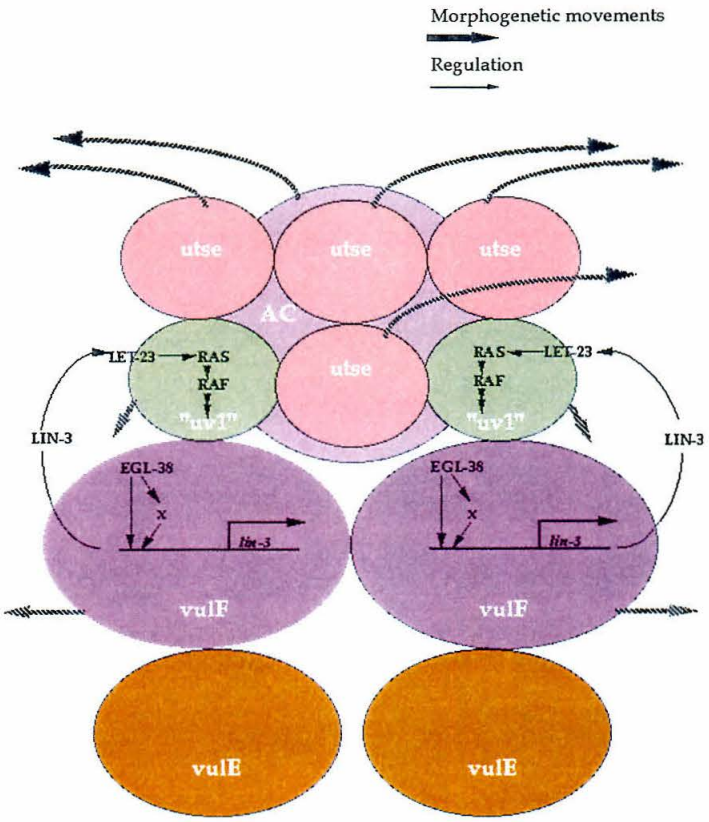

A
B
vulF
vulF
C
D

Figure 4. A model for the uv1 cell specification by a RAS-RAF dependent signaling pathway during development of the uterine-vulval connection. Each circle represents a specific cell with the name indicated in white. Arrows outside the cells indicate the direction toward which cells will move upon induction by a signaling pathway. EGL-38 acts directly or indirectly upstream of *lin-3* as a positive regulator in the vulF cells. Expression of *lin-3* in the vulF cells will then specify the uv1 cell fate through a RAS-RAF dependent signaling pathway in a cell non-autonomous manner.


Morphogenetic movements
Regulation
utse
utse
utse
AC
utse
LET-23
RAS
RAF
"uv1"
RAS
LET-23
RAF
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LIN-3
LIN-3
EGL-38
X
lin-3
EGL-38
X
lin-3
vulF
vulF
vulE
vulE

Table 1.

Specification of the uv1 cell fate by 1° vulval tissue occurs late in uterine development.

Genotype	Ablation	% of uv1 to utse transformation	Number of animals
+	----	0	20
let-23(sy97)	----	100	12
+	P(3-8).p	100	6
+	P6.p descendants at 2 cell stage	100	4
+	P6.p descendants at 4 cell stage	75	4
+	F lineages of P6.p at 4 cell stage	25	8
+	E lineages of P6.p at 4 cell stage	0	9
+	P(3,4,5,7,8).p	0	8
+	anchor cell at 4 cell stage	5	11
+	anchor cell at 4 cell stage	5	11

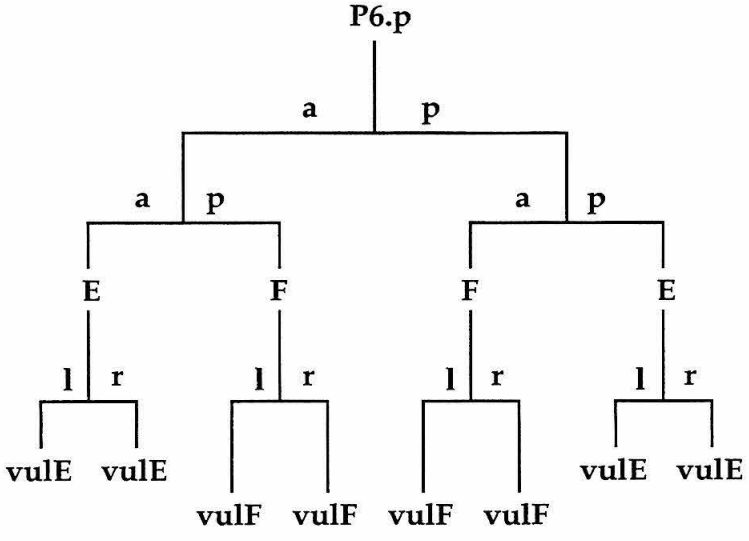

Table 1.

All ablations were done during the L3 stage. Pn.p designates the VPCs at the one cell stage; 2-cell stage= daughters of the VPCs; 4-cell stage= granddaughters of the VPCs. Beneath the Table is a schematic representation of 1° vulval cells and their lineal origins; adapted from Sulston and Horvitz (1977) [44]. The debris was not cleared in some of the ablation experiments; thus, these data demonstrate a requirement for the ablated cells at the indicated time points, but do not permit definitive conclusions regarding the ability of adjacent cells to replace them.

Table 2.

LET-23-mediated signaling is required for uv1 cell specification.

Genotype	% of specified uv1 cells [a]	Average number of VPCs undergoing vulval differentiation
a. +	100 (n=20)	3.0 (n=20)
b. *sli-1(sy143)*	100 (n=22)	3.0 (n=20)[b]
c. *gap-1(n1691)*	100 (n=20)	3.0 (n=21)
d. *let-23(sy97)*	0 (n=12)	0.0 (n=20)[b]
e. *let-23(sy97); sli-1(sy143)*	71 (n=28)	2.9 (n=131)[c]
f. *let-23(sy97); gap-1(n1691)*	10 (n=30)	2.7 (n=48)
g. *let-23(n1045)*	17 (n=20)	2.5 (n=20)[b]
h. *let-60(n2021)*	60 (n=20)	2.3 (n=20)
i. *lin-45(sy96)*	7 (n=20)	1.4 (n=24)
j. *lin-1(n1790)*	0 (n=12)	2.5 (n=15)

Extent of uv1 and vulval differentiation in certain LET-23 mediated signaling pathway mutants. The number values represent percentage of specified uv1 cells and average number of VPCs undergoing vulval differentiation per animal; 3.0 is wild-type level. n=number of animals scored.

[a] Data scored from only animals with a morphologically normal vulva in the mid-late L4 stage with the exception of *let-23(sy97)*.

[b] Data from Jongeward et al. (1995) [18].

[c] Data from Yoon et al. (1999) [45].

Full genotypes:

a, N2 (wild-type); b, *sli-1(sy143)*; c, *gap-1(n1691)unc-2(e55)*; d, *let-23(sy97)unc-4(e120)*; e, *let-23(sy97)unc-4(e120); sli-1(sy143)*; f, *let-23(sy97)unc-4(e120); gap-1(n1691)unc-2(e55)*; g, *let-23(n1045)unc-4(e120)*; h, *unc-24(e138)let-60(n2021)*; i, *lin-45(sy96)*; j, *lin-1(n1790)*

Table 3.

RAS and RAF are involved in uv1 cell fate specification.

	expressing dominant negative RAS variant in the mid L3 stage	*lin-45(sy96)* with wild-type vulval induction
Number of animals displaying wild-type vulval development	26[a]	20[b]
Number of animals displaying abnormal vulval development	7	13
Specified uv1 cell fate	50 %	8 %
Laid eggs at the adult stage	85 %	85%

[a] Animals were judged to have a wild-type vulval morphology by three criteria: wild-type number of vulval cells; normal cell lineages by anatomy[c]; characteristic physical appearance[d].

[b] Animals having wild-type vulval morphology have to match four criteria, wild-type number of vulval cells, normal cell lineages by anatomy, characteristic physical appearance and correct marker gene expression[e,f].

[c] We refer to the group of cells descended from a single VPC as a lineage; in the wild-type animal, the vulva is formed by one 1° lineage flanked by two 2° lineages. After two rounds of Pn.p cell division, we can easily distinguish three cell types (T, L and N) by Pn.pxx nuclear division (transverse "T" vs. longitudinal "L" vs. no division "N"); x refers to both progeny [46]. The two types of lineages that generate vulval cells are 1° ([TTTT]) and 2° ([LLTN] or [NTLL]).

[d] In the wild-type animal, after the last round of Pn.p cell division, cells of a canonical 1° lineage move dorsally and detach from the ventral cuticle. By the mid-L4 stage, they form a symmetric arch and separate anterio-posteriorly and left-right to create a hole so that eggs can pass through it. The 2° lineage is

asymmetric: the distal cells attach to the ventral cuticle; the proximal cells detach and migrate dorsally. By the mid-L4 stage, they form a characteristic structure (Figure 1C).

[e] We used *egl-17::GFP* expression as a marker to reflect a specific early 1° vulval property and a specific late 2° vulval property [32]. *egl-17::GFP* is expressed in the 1° vulval lineages during early vulval development (mid L3) and C and D lineages of the 2° vulval lineage in the mid-L4 stage.

[f] Only seven out of twenty animals have been followed for the expression of *egl-17::GFP* in the 1° and 2° vulval lineages.

Table 4.

***let-23(sa62)* is epistatic to *egl-38(n578)* with respect to uv1 cell specification.**

Genotype	2 uv1	1 uv1	0 uv1	% of specified uv1 cells	Number of animals
a. *let-23(sa62)*	21	0	0	100	21
b. *egl-38(n578)*	4	8	15	30	27
c. *let-23(sa62); egl-38(n578)*	32	3	0	96	35

Extent of uv1 differentiation in *let-23(sa62)*, *egl-38(n578)* and *let-23(sa62); egl-38(n578)* strains. In wild-type animals, there are four uv1 cells (left and right anterior to the presumptive vulval opening, and left and right posterior). Number of animals represent the number of animal sides followed. Specified uv1 (%) indicates the percentage of specified uv1 cells relative to the wild-type (100%).

Full genotypes:

a, *let-23(sa62); him-5(e1490)*; b, *dpy-20(e1282) egl-38(n578)*; c, *let-23(sa62); dpy-20(e1282) egl-38(n578); him-5(e1490)*.

Appendix 1

The RING finger/B-Box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in *Caenorhabditis elegans*

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The RING finger/B-Box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in *Caenorhabditis elegans*

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Context-dependent gene silencing is used by many organisms to stably modulate gene activity for large chromosomal regions. We have used tandem array transgenes as a model substrate in a screen for *Caenorhabditis elegans* mutants that affect context-dependent gene silencing in somatic tissues. This screen yielded multiple alleles of a previously uncharacterized gene, designated *tam-1* (for tandem-array-modifier). Loss-of-function mutations in *tam-1* led to a dramatic reduction in the activity of numerous highly repeated transgenes. These effects were apparently context dependent, as nonrepetitive transgenes retained activity in a *tam-1* mutant background. In addition to the dramatic alterations in transgene activity, *tam-1* mutants showed modest alterations in expression of a subset of endogenous cellular genes. These effects include genetic interactions that place *tam-1* into a group called the class B synMuv genes (for a Synthetic Multivulva phenotype); this family plays a negative role in the regulation of RAS pathway activity in *C. elegans*. Loss-of-function mutants in other members of the class-B synMuv family, including *lin-35*, which encodes a protein similar to the tumor suppressor Rb, exhibit a hypersilencing in somatic transgenes similar to that of *tam-1* mutants. Molecular analysis reveals that *tam-1* encodes a broadly expressed nuclear protein with RING finger and B-box motifs.

[*Key Words*: TAM-1; Chromatin Silencing; RING finger; *C. elegans*; Rb; RAS Pathway]

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Eukaryotes have evolved a number of concerted mechanisms that allow extended domains of individual chromosomes to be rendered inaccessible to the transcription apparatus. The use of such gene silencing mechanisms requires two different mechanistic operations. First, a cell must choose specific regions of the genome for silencing. Second, a silent state must be enforced. The most surprising feature of chromatin silencing (and the feature that distinguishes silencing from more localized modulation of gene expression) is the substantial distance at which silencing effects are seen. One striking example of this is the inactivation of the human X chromosome, which extends from a single locus (Xist) throughout a chromosome of 10^8 bases (Riggs and Porter 1996).

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The silencing apparatus can apparently be recruited by a variety of distinct features at the DNA sequence level. In many cases, a specific sequence element in DNA can be identified as necessary and sufficient for silencing of a surrounding domain. Examples of silencing effects with a clear nucleation point include telomere and mating-type silencing in yeast (Grunstein 1998; Haber 1998). In these cases, binding of a multiprotein silencing complex to the *cis*-silencing site is thought to nucleate an extended change in local chromatin structure.

Heterochromatin in many organisms includes regions of highly repetitive structure, and extensive speculation has attempted to link this repetitive structure to silencing properties of heterochromatin (Henikoff 1998). A potentially related class of silencing effects has been inferred from studies with transgenic fungi (Selker 1997), plants (Assaad et al. 1993; Ye and Signer 1996) and animals (Garrick et al. 1998). In these cases, a sequence that can be active as a single-copy locus in the genome becomes partially or completely inactive when repeated.

Strong effects generally require that the repeats are present at a single site in the genome, often as a tandem repeat. The identification of tandemly repeated sequences as appropriate for silencing could conceivably occur by two distinct mechanisms. In the first model, a weak *cis*-silencing element might be present in each member of the tandem repeat. In this case, the repetition would initiate silencing by juxtaposing several copies of the weak *cis*-silencing element. Alternatively, the organism could possess a mechanism allowing repeated structures to be recognized by virtue of repeated character and not of any specific sequence. The latter seems to be the situation in *Neurospora* (Selker et al. 1987; Cambareri et al. 1989; Selker 1997) and *Ascobolus* (Goyon and Faugeron 1989; Rossignol and Faugeron 1994), whereas the limited number of transgenic constructs tested for repeat-dependent silencing in the other systems leave both models open.

Despite the apparently conserved ability to recognize certain repeated sequences in DNA, there are differences between biological systems in the consequences of repetition. *Ascobolus*, *Neurospora*, mammals, and plants respond to repeated DNA structures by localized methylation of DNA (although *Neurospora* has the additional consequence of promoting point mutations in the DNA). Repeated structures in DNA in *Drosophila* produce variegated or mosaic expression without any methylation of DNA (Dorer and Henikoff 1994,1997).

In *Caenorhabditis elegans*, transgenes are generally carried as heritable extrachromosomal structures consisting of long tandem arrays with several hundred copies of the injected DNA (simple arrays) (Stinchcomb et al. 1985; Mello et al. 1991). Although expression (and phenotypic rescue) by such tandem arrays is readily observed, a difference in transcriptional activity compared with the endogenous locus is seen. The activity is frequently mosaic and expression levels (per copy) are frequently much lower than for the endogenous gene (Mello and Fire 1995). This difference is not a simple consequence of the extrachromosomal nature of the simple arrays; transgenes can be integrated into the chromosome by γ-irradiation producing stable transgenic lines, and these integrated arrays can still exhibit mosaic expression (e.g., Krause et al. 1994; J. Hsieh and A. Fire, unpubl.). Another factor that may affect the expression of simple arrays is the repetitive character inherent in the long tandem array. Consistent with this hypothesis, silencing of certain germ-line and somatic expression constructs can be relieved when the plasmid DNAs are cotransformed with an excess of *C. elegans* genomic DNA as carrier, producing a more complex array (Kelly et al. 1997; S. Xu, and A. Fire, unpubl.). To avoid specific assumptions about the role of particular sequences or general repeated structures in the silencing of tandem arrays in *C. elegans*, we will use the term context-dependent gene silencing. This paper describes the genetic identification and molecular characterization of one component that acts to attenuate the context-dependent silencing mechanism affecting tandem array transgenes in *C. elegans*.

Results

Isolation of modifiers of context-dependent gene expression

To identify factors responsible for context-dependent gene silencing, we screened for mutations that altered the activity of transgenes present in tandemly repeated arrays. We started with a stably integrated *myo-3::gfp* transgene expressing uniformly in all bodywall muscles (*ccIs4251*; Fire et al. 1998b). After mutagenesis with ethylmethanesulfonate (EMS), ~4500 mutagenized gametes were screened. Four putative mutant lines were recovered in which most, if not all, the animals have reduced-GFP expression (Fig. 1A, B). These strains were outcrossed to determine whether the reduced-GFP expression resulted (1) from changes in the transgene array, or (2) from (potentially interesting) mutations in endogenous genes. One mutation appeared to be dominant and linked to the transgene array; this could have reflected a change in copy number or in the structure of the array and was not analyzed further.

The three reduced-GFP expression strains were shown to contain chromosomal mutations (*cc566*, *cc567*, and *cc587*). All three were recessive and showed a temperature-sensitive effect with the decrease in *myo-3::gfp* expression (relative to the parent line), most pronounced at 25°C. At 16°C, the mutations had no observable effect on GFP levels. Because the mutant effects were temperature sensitive, all of the experiments were carried out at 25°C. Genetic mapping placed the three mutations on chromosome V. Complementation tests showed the three mutations to be allelic. The corresponding gene has been named *tam-1* (tandem-array modifier). The magnitude of the *tam-1*(*cc567*) effect on the *myo-3::gfp* transgene was estimated by optical quantitation of fluorescence to be ~18-fold (Fig. 1C).

The endogenous myo-3 *gene is not significantly affected by* tam-1

Alteration in the expression of the endogenous *myo-3* locus in a *tam-1* background was first tested genetically (Table 1). In an *unc-54* null mutant background, loss of one copy of the endogenous *myo-3* locus dramatically increases the severity of the muscle phenotype (Waterston 1989). *unc-54; tam-1* double mutants do not show a more severe phenotype (compared with *unc-54* alone), indicating that expression of the endogenous *myo-3* locus in a *tam-1* background is within twofold of wild type. Immunofluorescence experiments with a MYO-3 antibody similarly showed a lack of difference between and *tam-1* mutant animals (data not shown).

Transgene silencing in tam-1 *mutants depends on chromosomal context*

We took advantage of the ability to manipulate the transgene context as a means to investigate the apparent ability of *tam-1* to distinguish between transgene arrays and

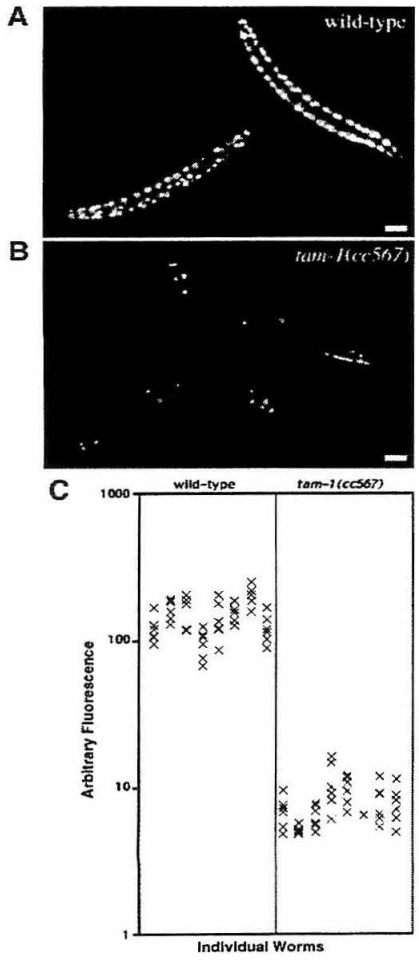

Figure 1. *tam-1* effects on a repetitive *myo-3::gfp* transgene. An integrated *myo-3::gfp* transgene was examined in wild-type and *tam-1(cc567)* mutant backgrounds. (*A*) Wild-type and (*B*) *tam-1(cc567)* animals photographed under fluorescent illumination showing GFP in bodywall muscles (the array produces GFP in both nuclear and mitochondrial compartments). Scale bars, 55 μm. Quantitative measurements of GFP fluorescence in wild-type and *tam-1(cc567)* mutant backgrounds. Differences in fluorescence level were most striking in the central region of the animal; thus quantitations were carried out in bodywall muscle nuclei in this region. Eight animals were analyzed for each genetic background. Fluorescence was quantitated for six nuclei in each animal with a Nikon U-III multipoint sensor system. Linearity of fluorescence measurements in the range of assay was confirmed by a series of neutral density filters. (wild-type mean= 140.9, wild-type (S.E.M.)= 5.9; *tam-1* mean = 7.2, *tam-1* S.E.M. = 0.4). A second measure of *tam-1* effects on the expression of a repeated transgene is observed with a *lin-3* transgene (*syIs1*). The average induction of vulval precursor cells (VPCs) caused by the expression of *syIs1* in wild type is 5.8 VPCs induced (n = 20), however, in *tam-1(sy272)*, there were 3.2 VPCs induced (n = 1 0) (data not shown).

endogenous loci. We used *myo-3* as a target for these assays, as earlier experiments (above) had shown that *myo-3::gfp* transgenes were affected, whereas the endogenous locus was not. The original *ccIs4251* transgene had been produced under a standard set of conditions that produce a highly repetitive tandem array carrying several hundred copies of the injected plasmid DNAs. We considered that differences between endogenous and transgene loci could have reflected (1) a difference in the genomic context of the endogenous gene and chromosomal locus, or (2) a difference between the *gfp* fusion and endogenous gene. To distinguish between these possibilities, we examined a novel type of transgene (Kelly et al. 1997) in which the incoming plasmid DNA was cotransformed with an excess of genomic DNA from *C. elegans*. The latter type of transgene (called a complex array) has a less repetitive structure, and might thus be a closer approximation to the native chromosomal context. Three new *myo-3::gfp* transgenes were produced in which the identical promoter was fused to *gfp*, but, in this case, was diluted 50- to 100-fold with fragmented genomic DNA. These complex arrays were strongly expressed and showed no difference in *tam-1*(+) versus *tam-1*(−) genetic backgrounds (Fig. 2A,B). Taken with the strong effect on simple arrays, these data indicate that *tam-1* effects on transgene activity are context dependent.

tam-1 *mutants have a silencing effect on many different repetitive transgenes*

The overexpression of a repetitive *lin-3* transgene (*syIs1*) results in a fully penetrant multivulva (Muv) phenotype and provides an alternative screen for decreased transgene activity (*lin-3* encodes a diffusible signal for vulval induction, Horvitz and Sulston 1980; Hill and Sternberg 1992) . From a screen of 73,000 EMS-mutagenized gametes, 15 additional alleles of *tam-1* were recovered. These mutants mapped to chromosome V and failure to complement *tam-1(cc567)* was observed. A representative allele of this group (*sy272*) also decreased the activity of the repetitive *myo-3::gfp* transgene, whereas a representative of the original group (*cc567*) showed the ability to decrease the activity of the *lin-3* transgene. As with the effects on *myo-3::gfp*, the *tam-1(sy272)* effect on a *lin-3* transgene was temperature dependent: strong suppression was seen at 25°C, whereas only limited suppression was observed at 16°C. The distinct expression patterns of *lin-3* (somatic gonad) and *myo-3* (bodywall muscle) suggested that *tam-1* might have effects on gene expression in many or all tissues. To test this hyposthesis, the expression of additional repetitive transgenes was analyzed in *tam-1* mutants (Table 2A). We observed a decrease in the expression of a number of diverse reporter constructs with different control signals and patterns of tissue specificity (including mesodermal, endodermal, and neuronal tissues). In addition, effects were seen with both *lacZ* and *gfp* transgenes.

As shown in Table 2B, not all reporter transgenes were strongly affected by *tam-1*. No obvious characteristic

Table 1. *Assays for* tam-1 *modulation of endogenous* myo-3 *function*

Genotype	Phenotype	Viability	Reference
unc-54(0) / *unc-54(0)*	paralyzed as adult	yes	Brenner (1974); Epstein et al. (1974)
myo-3(st378) / *myo-3(st378)*	L1 arrest	no	Waterston (1989)
unc-54(0) / *unc-54(0)* + *myo-3(st378)*	paralyzed after hatch; reaches adulthood	yes	Waterson (1989)
unc-54(0) *myo-3(st378)* / *unc-54(0)* *myo-3(st378)*	paralyzed as embryo	no	Waterston (1989)
unc-54(0) *tam-1(cc567)* / *unc-54(0)* *tam-1(cc567)*	paralyzed as adult [looks like *unc-54(0)* alone]	yes	this work

was shared between the transgenes in which we saw no *tam-1* effect. It is interesting, however, that several ubiquitously expressed transgenes (*let-858, rps-5*) appeared to be unaffected. Two neuronally expressed *gfp* fusions were also in this category. This may not be a universal property of neuronal expression: *tam-1* had strong effects on expression of *egl-15::gfp*, *lin-11::lacZ*, *gpa-1::lacZ*, and *mab-5::gfp* in neuronal tissues.

tam-1 *effects on endogenous genes*

Although *tam-1* mutants were fertile, they were not wild type. Brood sizes were considerably reduced. In three independent alleles (*cc567*, *cc587*, and *sy272*), average brood sizes at 25°C ranged from 55.8–105.1, compared with 204 for wild-type and 187 for the parental (*gfp* fusion) strain. *tam-1* mutants also showed defects in X chromosome segregation, as evidenced by a high incidence of males (XO animals) derived from XX parents. Male frequencies for the three alleles varied between 0.5% and 8.6% compared with the <0.2% incidence of spontaneous males in wild-type animals and in the parent strain PD4251.

Sensitized genetic assays were used to test for *tam-1* effects on several endogenous genes (Table 3). No effects (i.e., less than a twofold difference) was seen for *unc-22* (data not shown), or for gain-of-function alleles of *let-23* and *let-60*. In a number of other sensitized genetic assays, we found modest enhancement by *tam-1*, in each of these cases (*lin-2*, *let-2*, *lin-3*), a partial loss-of-function allele gave a more severe phenotype in a *tam-1*(−) genetic background. For example, *tam-1* reduced vulval differentiation in a *lin-3* hypomorphic strain *e1417* to the level of the *e1417*/null hemizygote; thus, there was an approximately twofold effect. Similar arguments indicated that the magnitude of the *tam-1* effect on *lin-2* and *let-2* activity was also approximately twofold.

tam-1 *has properties of a class-B synthetic Muv gene*

Two *C. elegans* genes were recently identified (*lin-35* and *lin-53*) with a redundant role in the modulation of RAS signaling (Lu and Horvitz 1998). Mutations in these genes, like *tam-1*, are viable and have modest genetic interactions with extragenic mutations. *lin-35* and *lin-53* encode proteins similar to Rb and Rb-associated protein p48, respectively. *lin-35* and *lin-53* are members of a larger class of genes, called class-B synMuv genes, which are defined by the observation that null or hypomorphic mutations lead to little or no phenotypic effect unless a second mutation is present in a distinct set of genes termed the class-A synMuv genes (Horvitz and Sulston 1980; Ferguson and Horvitz 1989). Double mutants carrying a class-A and a class-B allele show phenotypes characteristic of hyperactivity of the RAS-signaling pathway (most notably a Muv phenotype). Numerous class-A and class-B synMuv genes have thus been identified by these studies, with the working model that these sets of genes define two redundant pathways inhibiting EGFR–RAS-signaling activity.

Given the fact that *lin-35* and *lin-53*, two class-B SynMuv genes, encode factors shown to mediate chromatin repression in other systems (Lu and Horvitz 1998; Luo et al. 1998), we investigated the relationship of *tam-1* to these groups of genes. Double mutants carrying a *tam-1* mutation and a mutation in *lin-15A* (a class-A synMuv gene) showed a Muv phenotype at 25°C (Table 4; Fig. 3A). In contrast, *tam-1*(*cc567*); *lin-15B*(*n744*) double mutants did not have a Muv phenotype (Table 4). Two other class-A synMuv genes were analyzed for interactions with *tam-1*. Double mutants carrying *tam-1*(*cc567*) and the class-A synMuv mutation *lin-38*(*n751*) showed a synthetic multivulval phenotype, whereas no such phenotype was observed in *tam-1*(*cc567*); *lin-8*(*n111*). The latter result may indicate a functional division within the A class of synMuv genes, although it should be noted that differences in penetrance of the Muv phenotype in different A/B combinations have been observed previously (Ferguson and Horvitz 1989; Thomas and Horvitz 1999). In any case, it is clear from the interaction with *lin-15A* and *lin-38* that *tam-1* shares functional properties with the previously identified class-B synMuvs.

An additional connection between *tam-1* and the class-B synMuv family comes from analysis of tandem array transgene activity in previously characterized class-B synMuv mutants. A mutation eliminating the RB

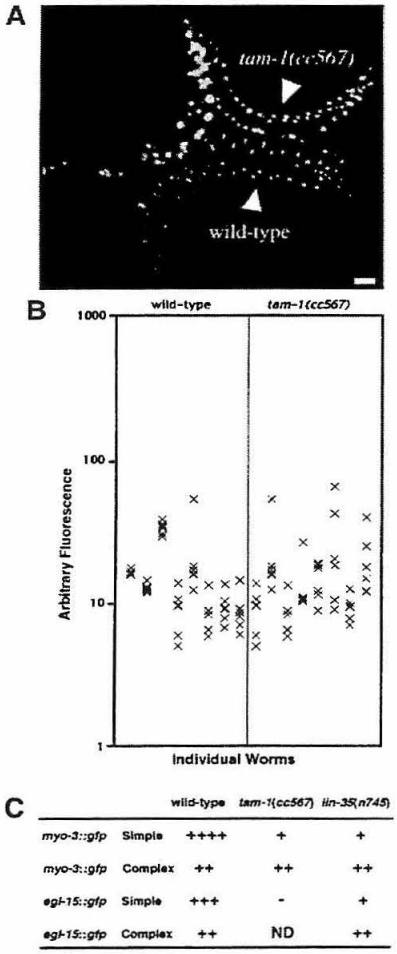

Figure 2. Assessment of *tam-1* effects in a nonrepetitive context. A nonrepetitive transgene array (*ccEx6172*) was compared in wild-type and *tam-1(cc567)* mutant backgrounds. (*A*) Wild-type and *tam-1(cc567)* animals photographed under fluorescent illumination showing GFP in bodywall muscles (the array produces GFP in nuclei). Scale bar, 55 µm. (*B*) Quantitation of fluorescence was carried out in six nuclei in the central region of the body for each of eight animals as in Fig. 1B. (wild-type mean = 15.2, wild-type S.E.M. = 1.4; *tam-1* mean = 15.6, *tam-1* S.E.M. = 1.7). (*C*) Analysis of repetitive (simple) and nonrepetitive (complex) transgene activity in wild-type, *tam-1(cc567)* and *lin-35(n745)* backgrounds. Transgenes are as described in Materials and Methods.

homolog Lin-35 was tested by simple and complex arrays of *myo-3::gfp* and of *egl-15::gfp* (Fig. 2C). The two simple transgene arrays were both silenced in a *lin-35(n745)* background, whereas no effect of *lin-35(n745)* on either complex array was observed. Five additional class-B mutants (*lin-9(n112)*, *lin-15B(n744)*, *lin-36(n766)*, *lin-51(n770)*, and *lin-52(n771)*) were tested for silencing of a simple transgene array; all except for *lin-36(n766)* were found to produce a transgene-silencing phenotype similar to *tam-1* (Fig. 3B,C; data not shown). The class-A synMuv mutations *lin-8(n111)*, *lin-15A(n767)*, and *lin-38(n751)* showed no transgene-silencing phenotype.

Molecular identification of tam-1

The *tam-1* locus was mapped more precisely to a small region of chromosome V very close to the gene *unc-46* (Fig. 4A). On the basis of the known position of *unc-46* on the physical map, (E. Jorgensen, pers. comm.) we obtained cosmids that covered this region, injected pools of overlapping cosmids, and assayed for rescue of *tam-1* effects on *myo-3::gfp* expression. We found that cosmid F26G5 rescued the decreased-GFP phenotypes of three *tam-1* alleles (Fig. 4B). Testing of subcloned fragments from this cosmid led to the identification of an 8.6-kb fragment with rescuing activity (Fig. 4C). A single gene

Table 2. tam-1 *effects on reporter transgene activity*

	Construct	Relative intensities of reporters: expression pattern	wild-type	*tam-1*
A	*myo-3::gfp*	body-wall muscle	++++	+
		vulval muscle	+++	+
	unc-54::gfp	body-wall muscle	+++	+
	hlh-1::gfp	body-wall muscle	++	–
	myo-2::gfp	pharyngeal muscle	++++	++
	F22B7.9::gfp	gut	++	–
	egl-15::gfp	vulval muscle	+++	–
		gut	+	–
		neurons near head	+	–
	lin-3::lacZ	anchor cell	+++	+
	gpa-1::lacZ	head neurons	+++	+
		plasmid neurons	+++	+
	lin-11::lacZ	2° vulval lineages	++	–
		VC motor neurons	+++	–
		ventral uterine π cells and their progeny	++	–
	mab-5::gfp	posterior region	+	–
		neurons near tail	+	–
B	*let-858::gfp*	all somatic nuclei	+++	+++
	rps-5::gfp	all somatic nuclei	+++	+++
	glr-1::gfp	subset of neurons	+++	+++
	unc-119::gfp	many neurons	+++	+++

Expression of tandemly repeated transgenes in wild-type and *tam-1* mutants. GFP transgenes were examined in *tam-1(cc567)*, and *lacZ* transgenes were examined in *tam-1(sy272)*. Number of plus signs (+) indicate relative intensity of GFP fluorescence or *lacZ* signal. (–) No observable signal. In cases where no expression was observed in *tam-1* mutants, an outcross was performed to confirm the reappearance of reporter expression. (*A*) *tam-1* effects were seen on a number of different reporter constructs. (*B*) Other transgenes showed little or no effect in *tam-1* mutants. Additional *myo-3::gfp* and *rps-5::gfp* transgenes (independently derived) were tested in wild-type and *tam-1(cc567)* backgrounds and showed similar results (data not shown).

Table 3. tam-1 *effects on endogenous gene activity*

Genotype	Phenotype	Statistical significance
let-2(mn114)	sterile or semisterile adults (n = 8)	
let-2(mn114); tam-1(sy272)	L1 or L2 lethal (n > 20)	
lin-2(n768)	3.25 VPCs induced (n = 26)	P < 0.0001
lin-2(n768); tam-1(sy272)	1.81 VPCs induced (n = 18)	
lin-3(e1417)	0.66 VPCs induced (n = 102)	P < 0.0001
lin-3(e1417); tam-1(sy272)	0.29 VPCs induced (n = 92)	
let-23(sa62)	3.42 VPCs induced (n = 6)	P = 0.17
let-23(sa62); tam-1(sy272)	4.4 VPCs induced (n = 4)	
let-60(n1046)	3.97 VPCs induced (n = 16)	P = 0.0029
let-60(n1046); tam-1(sy272)	4.93 VPCs induced (n = 32)	

Phenotypes are described for a number (n) of animals for each of the genotypes. The state of vulval morphogenesis was assessed by counting induced vulval precursor cells (normally three in wild type). Statistically significant differences between wild-type and *tam-1(sys272)* were observed in *lin-2(n768)*, *let60(n1046)*, and *lin-3(e1417)* backgrounds. The average induction of VPCs for *lin-3(e1417)/lin-3(n1059)* is 0.34, n = 22 (P.W. Sternberg, unpubl.). For statistical analysis, a Fisher's exact test was performed using Instat (GraphPad Software).

had been predicted in this region (*C. elegans* Sequencing Consortium 1998). When we created a frameshift in a unique *Aat*II site within the coding region, the resulting fragment was incapable of rescuing *tam-1(cc567)*. The mRNA structure predicted by the sequencing consortium (Fig. 4D) was correct, as determined by completing the sequence of a full-length cDNA clone obtained from Y. Kohara [pers. comm.; accession nos. C42160 (5′), C31634 (3′)]. Next, we identified the molecular lesions associated with three of the *tam-1* alleles (Fig. 4E). All three alleles resulted in nonsense mutations, with cc567 containing the most amino-terminal stop codon.

The predicted TAM-1 protein contains two cysteine-rich regions, a C3HC4 (RING finger) motif and a B-box motif. The RING finger and B-box motifs have been defined by primary sequence comparisons; although both domains may act as metal (zinc)-binding domains, there is not yet any basis to predict specific biochemical function (Saurin et al. 1996). *C. elegans* contains 90 RING finger proteins (*C. elegans* Sequencing Consortium 1998). Several *Drosophila* proteins with roles in global regulation of chromosome activity also contain RING finger motifs; these include the Posterior Sex Combs (PSC) and Suppressor Two of Zeste [Su(Z)2] proteins (which are involved in the maintenance of repressed transcriptional states by the polycomb family; Brunk et

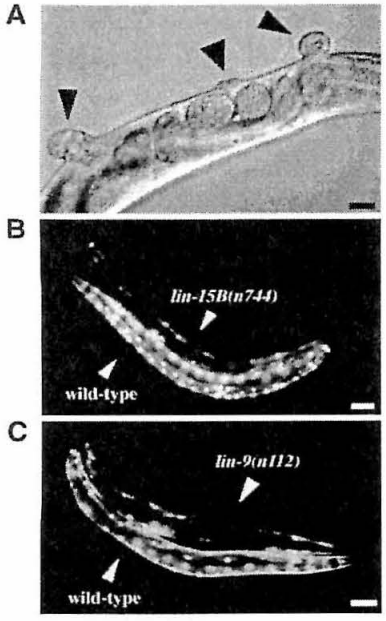

Figure 3. *tam-1* has properties of a class-B synMuv gene. (*A*) *lin-15A(n767); tam-1(cc567)* double mutants are Muv (arrowheads indicate the position of vulvae). Scale bar, 27.5 μm. Other class-B synMuv mutants have decreased transgene expression. (*B*) The expression of *ccIs4251(myo-3::gfp)* is examined in wild-type and homozygous *lin-15B(n744)* animals. Scale bar, 87.5 μm. (*C*) The expression of the same transgene is also examined in wild-type and homozygous *lin-9(n112)* animals. Scale bar, 87.5 μm.

Table 4. tam-1 *has properties of a class B synMuv gene*

Genotype	Phenotype
tam-1(cc567)	3 VPCs induced (n = 15)
lin-15A(n767)	3 VPCs induced (n = 15)
tam-1(cc567); lin-15A(n767)	4.9 VPCs induced (n = 20)
tam-1(cc567); lin-15B(n744)	2.9 VPCs induced (n = 15)

The average induction of vulval precursor cells for each genotype is shown. All counts of VPC induction were performed with L4 staged animals at 25°C. At 20°C, the frequency of multivulva animals in *tam-1(cc567); lin-15A(n767)* double mutants decreased considerably.

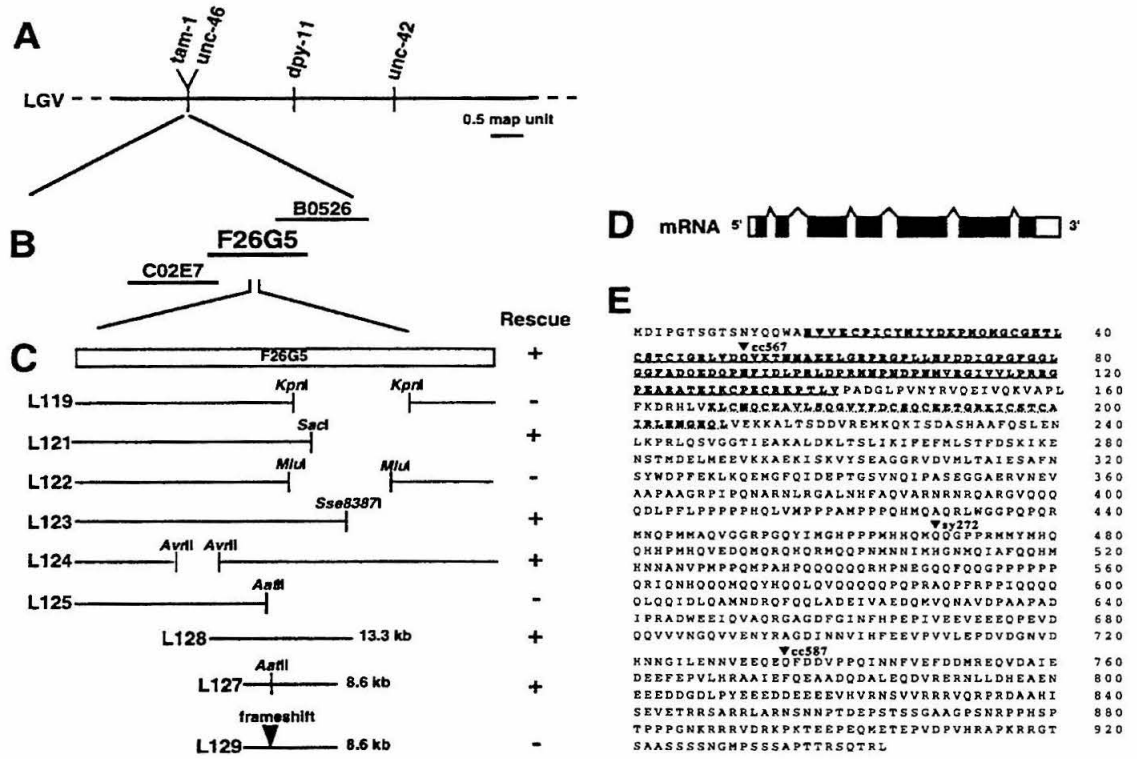

Figure 4. Molecular identification of *tam-1*. (*A*) Genetic mapping placed the *tam-1* locus to the left of the gene *unc-46*. (*B*) Pools of cosmids were assayed for rescue of the reduced-transgene-expression phenotype of *tam-1*(*cc567*). Cosmid F26G5 was found to rescue the decreased-GFP phenotype of *tam-1*(*cc567*); *ccIs4251*(*myo-3::gfp*). Rescue was also observed for two other *tam-1* alleles, *cc587* and *sy272*. (*C*) Deletion analysis of cosmid F26G5 led to the identification of an 8.6-kb fragment (L127) with rescuing activity. Analysis of the sequence of this fragment had predicted a single coding region (*C. elegans* Sequencing Consortium 1998). Creation of a frameshift in a unique *Aat*II site within this coding region abolished rescue. (*D*) A full-length cDNA was obtained from the Kohara laboratory. Sequencing of this cDNA confirmed that the *tam-1* mRNA sequence contains seven exons and six introns. (*E*) Amino acid sequence of TAM-1. Underlined are the RING finger (solid line) and B-box (broken line). Sequencing of *tam-1* alleles (*cc567*, *sy272*, and *cc587*) revealed in each case the conversion of a glutamine codon (CAA) to a nonsense codon (TAA). DNA from *tam-1* mutants was amplified by PCR; this was done in several segments covering a region from 288-bp upstream of the ATG to 440-bp downstream of the deduced *tam-1* translational stop. PCR products were directly sequenced, with mutations confirmed by sequencing products from at least two different PCR reactions. The three distinct mutations are indicated by arrowheads.

al. 1991), and MSL-2 (involved in sex-specific activation of the X chromosome in males; Zhou et al. 1995). Although these comparisons are intriguing, proteins containing RING finger and B-box motifs have been found in many additional biological processes (Fig. 5). The similarity of TAM-1 to other characterized RING finger and B-box proteins does not extend outside of these two domains.

TAM-1 protein is present in nuclei throughout development

We raised polyclonal antibodies against two nonoverlapping protein fragments from the carboxyl terminus of TAM-1. The two antisera gave identical patterns, with staining in the nuclei of most, if not all, somatic cells in wild-type embryos (Fig. 6A,B). The nuclear distribution was uniform, with staining beginning at about the 20-cell stage and persisting throughout development. There was also nuclear staining (albeit somewhat fainter) in larval and adult cells and germ-line and oocyte nuclei (Fig. 6C,D). Antibody specificity was verified by examining null mutant embryos, larvae, and adults (no detectable staining by either antisera).

tam-1 *is not essential for viability*

Several observations support the hypothesis that the viable chain-termination allele *tam-1*(*cc567*) is a null mutation: (1) *tam-1*(*cc567*) is predicted to result in transla-

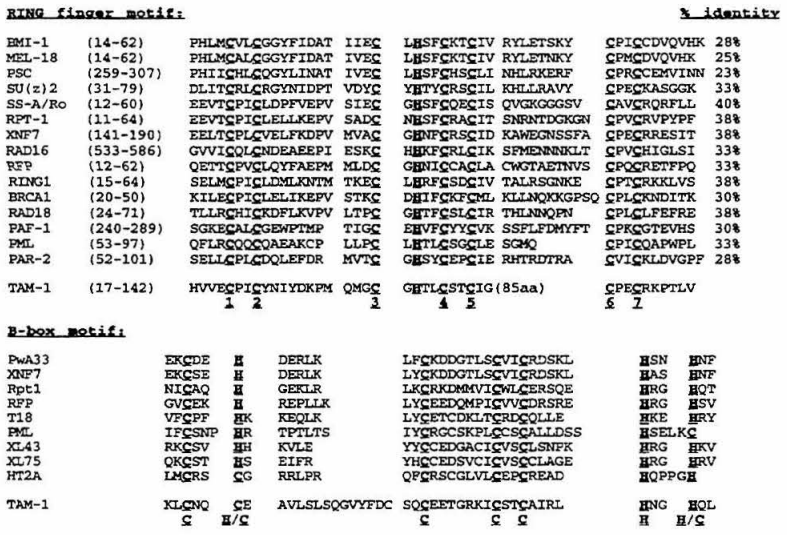

Figure 5. Sequence alignments of RING finger and B-box motifs of TAM-1 with related segments of other proteins. Conserved cysteine and histidine residues are underlined. A limited number of members of each family were chosen arbitrarily. GenBank contains >100 entries with RING finger motifs. Alignment of RING finger motifs: Numbering below each conserved cysteine refers to zinc binding ligands cysteine 1-cysteine 7 (Saurin et al. 1996) . Percent identity refers to just the RING finger domain. Alignment of B-box motifs: Sources for RING fingers and B-Box motifs used in this comparison are RAD18 (Jones et al. 1988); RPT-1 (Patarca et al. 1988); PSC, SU(Z)2 (Brunk et al. 1991); SS-A/Ro (Chan et al. 1991); XNF7 (Reddy et al. 1991); RAD16 (Bang et al. 1992); PML (Kastner et al. 1992); PAF-1 (Shimozawa et al. 1992); PwA33 (Bellini et al. 1993); MEL-18 (Ishida et al. 1993); PAR-2 (Levitan et al. 1994); BRCA1 (Miki et al. 1994); HT2A (Fridell et al. 1995); T18 (Miki et al. 1991; LeDoyerin et al. 1995); RFP (Cao et al. 1997); RING1 (Satijn et al. 1997); BMI-1 (Hemenway et al. 1998); and XL43, XL75 (Perrin and Lacroix 1998).

tional arrest after synthesis of only 50 amino acids; (2) Hemizygous [*tam-1*(*cc567*)/*nDf32*] animals showed a phenotype indistinguishable from *tam-1*(*cc567*)/*tam-1*(*cc567*) (data not shown); (3) double-stranded RNA interference (Fire et al. 1998b) with a segment of *tam-1* produced a nonlethal reduced-GFP phenotype identical to that of *tam-1*(*cc567*) (data not shown); (4) viable *tam-1* alleles arose at a high frequency (2.2×10^{-4} after EMS mutagenesis) close to reported knockout frequencies for *C. elegans* (Brenner 1974); and (5) there was no detectable staining with either TAM-1 antibody in *tam*(*cc567*) (data not shown).

Discussion

tam-1 *and context-dependent gene silencing*

We have presented evidence that TAM-1, a novel RING finger and B-Box factor, plays a role in modulating context-dependent gene silencing. *C. elegans* transgenes are often subject to silencing when present in tandemly repeated array structures (simple arrays). This silencing is particularly evident in the germ line (Kelly et al. 1997), but can also be observed in the soma of *C. elegans* (Okkema et al. 1993; Krause et al. 1994). This silencing appears to be context dependent because it can be attenuated or abolished by placing the transgene in a less repetitive context (complex arrays).

In *tam-1* null mutants, the silencing of transgenes present in tandemly repeated arrays was potentiated. Two comparisons suggest that *tam-1* effects on gene expression are sensitive to chromosomal context. First, the dramatic reductions in activity seen with simple arrays (e.g., 18-fold for a canonical *myo-3*::*gfp* array) were not seen with the corresponding endogenous loci (e.g., no effect on the endogenous *myo-3* locus in an assay in which a twofold effect would have been observed). Second, complex arrays are much less susceptible to silencing in a *tam-1* null mutant background. This suggests that the silencing activity in *tam-1* mutants has some degree of preference for repetitive transgene arrays. Hence, the wild-type TAM-1 product appears (directly or indirectly) to be attenuating context-dependent silencing.

Although *tam-1* had no discernible effect on the endogenous *myo-3* locus, a number of genes showed an apparent decrease in expression levels in a *tam-1* mutant background. Although the mechanistic basis for these modest effects was not investigated further, one possibility is that all effects of *tam-1* might reflect a direct potentiation of context-dependent silencing; in this model, the silencing mechanism might have drastic activity for some regions of chromosomes (e.g., regions that might behave similarly to highly repeated transgene arrays) with a less evident effect on other regions (e.g., subtle or indirect effects on genes in partially repetitive chromosomal areas). An additional possibility is that *tam-1* might be a specific transcription factor regulating a small number of target genes, including one or more genes with a general role in context-dependent silencing. Clearly the above models are not mutually exclusive.

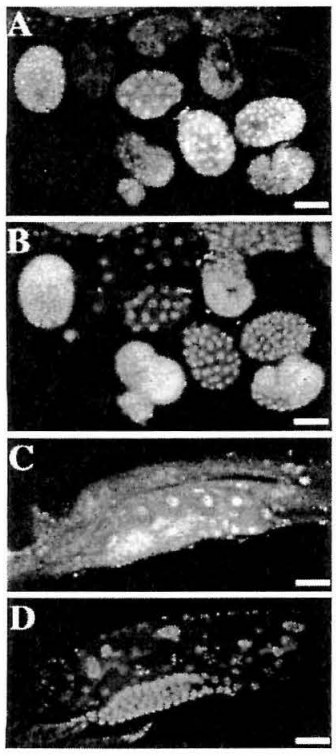

Figure 6. Immunofluorescence detection of TAM-1 protein in nuclei. (*A*) Embryo population and (*C*) an adult stained with mouse anti-TAM-1 and visualized with a secondary goat anti-Cy3 antibody. (*B*,*D*) Corresponding images of DNA localization, visualized with DAPI. Photos shown are from antisera to TAM-1 residues 594–712. Similar results were seen with antisera to TAM-1 residues 778–856. Signal in *C* has been augmented by electronic contrast enhancement. Scale bars, 27.5 μm.

A connection between context-dependent gene silencing and control of the RAS pathway?

Genes of the class-B synMuv family have been shown to play a role in the modulation of EGFR–RAS pathway activity during vulval specification. This role is redundant in that loss-of-function mutants in class-B genes show vulval specification defects (apparent hyperactivity of the RAS pathway) only if a second mutation in a class-A synMuv gene is present (Horvitz and Sulston 1980; Ferguson and Horvitz 1989; Clark et al. 1994; Huang et al. 1994). Although much of the genetic analysis of class-B synMuv activity has involved analysis of vulval specification, mutants have also been reported to exhibit defects in growth and fertility, suggesting additional functions for these genes outside the vulva (Ferguson and Horvitz 1989).

Our work extends this analysis in two ways. First, TAM-1 was shown to have properties of a class-B synMuv factor and also to modulate transgene reporter activity in a wide variety of nonvulval tissues including mesoderm (pharyngeal muscles, body muscles, and somatic gonad), endoderm (gut) and at least a subset of neurons (from expression of *egl-15*, *lin-11*, *gpa-1*, and *mab-5*). Second, we have observed effects of previously characterized class-B synMuv genes (*lin-9*, *lin-15B*, *lin-35*, *lin-51*, and *lin-52*) on transgene activity levels in extensive subsets of mesoderm. Although not all class-B synMuv genes have been examined, and not all cell types have been included in the reporter expression patterns that were tested, it appears likely that this family of genes might act in most or all tissues of the animal. Consistent with this hypothesis, *tam-1* and several other class-B synMuv genes encode proteins with broad expression patterns (Lu and Horvitz 1998; this work; L.Huang, J. DeModena, and P.W. Sternberg, unpubl.).

What are the natural targets of the class-B synMuv family? As noted above for *tam-1*, one possibility for the class-B SynMuv factors is that they form a relatively specific regulatory complex whose targets include both RAS pathway components and (independently) factors involved in the modulation of repetitive transgenes. Under these circumstances, there would be no direct link between RAS signaling and silencing of repetitive arrays. We favor the alternative hypothesis that some relationship exists between these two processes. In particular, our current working model is that the class-B synMuv factors modulate context-dependent silencing of a wide variety of biological targets; some of these targets may be endogenous genes that are present in areas of repetitive sequences or an otherwise inopportune genomic environment.

At some level, the effects of the class-B synMuv genes are likely to involve changes in the acetylation state of histones in chromatin. The strongest indication for this hypothesis comes from the ability to induce a class-B synMuv phenotype by selective disruption of histone deacetylase-1 (*hda-1*) function and from the existence of a complex containing LIN-35 (Rb), LIN-53 (p48), and HDA-1 (Lu and Horvitz 1998). It is not clear whether TAM-1 participates as a part of this complex, or whether the complex interacts directly with repetitive transgene arrays. The directionality of the class-B synMuv effect should be considered in evaluating the following: transgene expression is reduced in the absence of LIN-35 or TAM-1 function. Thus, if there were a direct interaction in wild-type animals, it would be (unexpectedly) a positive effect. In many cases, histone deacetylase complexes are thought to act as negative regulators of gene expression (Kuo and Allis 1998). An additional possibility is that the class-B synMuvs normally act to direct silencing factors to a defined set of targets not including tandem array transgenes; under these circumstances, class-B mutants might result in an excess of unused silencing factors and consequent hypersilencing of certain loci (including transgene arrays) with susceptibility to the process. A similar proposal involving differential partitioning of a limiting supply of generalized silencing factors was proposed by Grewal and Klar (1998) in their studies of chromosomal silencing in fission yeast.

Materials and methods

C. elegans *strains and culture*

Conditions for growth and maintenance were as described (Brenner 1974). Unless stated otherwise, experiments were conducted at 25°C. Wild-type N2 (var. Bristol) and the standard mutant strains listed below are as described by Brenner (1974), unless otherwise noted. The following genes and alleles were used: LG I, *unc-54(e190)*, *lin-35(n745)* *unc-13(e189)* (Ferguson and Horvitz 1989); LG II, *let-23(sa62)* *unc-4(e120)* (Katz et al. 1996), *lin-8(n111)* *dpy-10(e128)* (Ferguson and Horvitz 1989), *lin-38(n751)* (Thomas and Horvitz 1999); LG III, *lin-9(n112)* (Ferguson and Horvitz 1989), *lin-36(n766)* *unc-32(e189)* (Thomas and Horvitz 1999), *lin-51(n770)* *unc-32(e189)* (Thomas and Horvitz 1999), *lin-52(n771)* *unc-32(e189)* (Thomas and Horvitz 1999); LG IV, *dpy-20(e1282)*, *lin-3(e1417)* (Horvitz and Sulston 1980), *let-60(n1046)* *unc-31(e169)* (Ferguson and Horvitz 1985; Han and Sternberg 1990); LG V, *tam-1(cc566, cc567, cc587,* and *sy272)*, *unc-46(e177)*, *dpy-11(e224)*, *unc-42(e270)*, *dpy-11(e224) nDf32/eT1* (Park and Horvitz 1986); and LG X, *lin-2(n768)*, *let-2(mn114)* *unc-3(e151)*, *lin-15A(n767)* (Ferguson and Horvitz 1985), *lin-15B(n744)* (Ferguson and Horvitz 1985).

Plasmid constructs used in transgenes

pSAK-2 (*myo-3::Ngfp–lacZ*) contains the *myo-3* promoter driving *gfp* expression in all nonpharyngeal muscles; a nuclear localization signal (NLS) and *lacZ* sequences are appended to the reporter coding sequence, and lead to predominantly nuclear localization (Fire et al. 1998b).

pSAK-4 (*myo-3::Mtgfp*) contains the *myo-3* promoter driving *gfp* expression in nonpharyngeal muscles; the GFP has a mitochondrial localization signal (Fire et al. 1998b).

pPD96.02 (*unc-54::Ngfp–lacZ*) contains the *unc-54* promoter driving *NLS::gfp::lacZ* in nonpharyngeal muscles (Fire et al. 1998a).

pPD105.19 (*unc-54::Mtgfp*) contains the *unc-54* promoter driving *gfp* expression with a mitochondrial localization signal in nonpharyngeal muscles (Fire et al. 1998a).

pPD93.92 (*hlh-1::gfp*) is a translational fusion containing the *hlh-1* promoter and first exon driving *gfp* expression in myogenic precursor cells and bodywall muscles (derived from pPD37.48 (Krause et al. 1990) by substitution of *gfp* for *lacZ*).

pPD118.33 (*myo-2::gfp*) contains the *myo-2* promoter driving *gfp* expression in pharyngeal muscle (S. Xu and A. Fire, unpubl.).

pPD103.68 (*F22B7.9::gfp*) is a translational fusion driving *gfp* in the gut (S. Xu and A. Fire, unpubl.).

pNH#300 (*egl-15::gfp*) contains the *egl-15* promoter driving *gfp* expression in Vm1 vulval muscles (Harfe et al. 1998).

pRH9 (*lin-3*) is a *lin-3* genomic construct (Hill and Sternberg 1992).

pCC2 (*lin-3::lacZ*) is a translational fusion of the *lin-3* promoter and coding region before the first cytoplasmic domain driving *lacZ* expression in the anchor cell and many other tissues (Chang et al. 1999).

pjM1HG.11 (*gpa-1::lacZ*) contains the *gpa-1* promoter driving *lacZ* expression in plasmid neurons, PHA and PHB (J. Mendel and P.W. Sternberg, unpubl.).

pGF66 (*lin-11::lacZ*) is a translational fusion that drives *lacZ* expression in descendants of vulval precursor cells (P5.pp and P7.pa), VC motor neurons, and in the ventral uterine p cells and their progeny (Freyd 1991).

pCH22 (*mab-5::gfp*) contains the *mab-5* promoter driving *gfp* expression in cells of the posterior region (Hunter et al. 1999).

BK48 (*let-858::gfp*) contains a complete copy of the *let-858* gene tagged by in-frame insertion of a *gfp* cassette within the coding region, yielding *gfp* expression in all somatic nuclei (Kelly et al. 1997).

pPD129.51 (*rps-5::gfp*) contains the promoter for the gene T05E11.1 (encoding ribosomal protein S5) driving *gfp* expression in all somatic nuclei (J. Fleenor and A. Fire, unpubl.).

pG75#KP6 (*glr-1::gfp*) contains the *glr-1* promoter driving *gfp* expression in a subset of neurons (H. Hutter, pers. comm.).

pDP#MMUGD12 (*unc-119::gfp*) is a translational fusion containing the *unc-119* promoter and *unc-119* genomic region driving *gfp* expression throughout the nervous system (Maduro and Pilgrim 1995).

pRF4 (*rol-6 [su1006]*) carries a dominant allele of the gene *rol-6* that leads to an easily identified rolling phenotype.

pMH86 (*dpy-20[+]*) carries a wild-type copy of the gene *dpy-20* that provides a ready selection for transgene function in a *dpy-20(e1282ts)* background (Han et al. 1993; Clark et al. 1995).

Transgenic lines

In some cases (lines referred to as *Is*), the transgene arrays had been integrated by γ-ray mutagenesis. In all other lines (referred to as *Ex*), the transgene arrays remained extrachromosomal.

For each transgene, the comparison of wild-type with *tam-1(–)* and/or *lin(–)* backgrounds was made several generations after the strain had been established; at this point simple culture of the strains produced no evident instability in expression or transmission properties. Effects on transgene expression were observed in all animals from each affected population (>100 animals for each transgene).

Simple array lines were produced by injection of a mixture of the circular reporter plasmid and a circular selectable marker plasmid, with no other DNA added as carrier (Mello and Fire 1995).

ccIs4251 pSAK2(*myo-3::Ngfp–lacZ*), pSAK4(*myo-3::Mtgfp*), and pMH86 (Fire et al. 1998b). The multicopy tandem-array character of the *ccIs4251* transgene was confirmed by Southern blot analysis (showing ~100 copies each of pSAK2 and pSAK4; data not shown). *ccIs4251* was obtained following treatment with γrays and shows some crossover suppression activity in the center of chromosome I.

ccEx6188 pSAK2(*myo-3::Ngfp–lacZ*) and pRF4 (this work). A simple extrachromosomal transgene with expression levels comparable with the integrated *ccIs4251* array, *ccEx6188* was used to assay mutations on chromosome I (e.g., *lin-35*) for silencing effects.

ccIs9385 pPD96.02(*unc-54::Ngfp–lacZ*), pPD105.19(*unc-54::Mtgfp*), and pRF4 (Fire et al. 1998a).

ccIs7963 pPD93.92(hlh-1::gfp) and pRF4 (K. Dej and A. Fire, unpubl.).

ccIs4792 pPD95.29(*pes-10::gfp*), pPD118.33(*myo-2::gfp*), pPD103.68(*F22B7.9::gfp*), and pMH86 (M. Edgley, K. Liu, A. Fire, and D. Riddle, unpubl.)

ayIs2 pNH#300(*egl-15::gfp*) and pMH86 (Harfe et al. 1998).

syIs1 pRH9(*lin-3*) and an *unc-31* subclone (selectable marker) (Hill and Sternberg 1992; this work; J. Liu, unpubl.).

syEx241 pCC2(*lin-3::lacZ*), pMH86, and pSK+ as carrier (Chang et al. 1999).

syIs19 cpjM1HG.11(*gpa-1::lacZ*) and pMH86 (J. Mendel and P.W. Sterberg, unpubl.).

nIs2 pGF66(*lin-11::lacZ*) and pGF60(*lin-11* rescuing plasmid) (Freyd 1991).

muIs16 pCH22(*mab-5::gfp*) and pMH86 (Hunter et al. 1999).

ccEx7997 BK48(*let-858::gfp*) and pRF4 (Kelly et al. 1997).

ccEx8151 pPD129.51(*rps-5::gfp*) and pRF4 (J. Fleenor and A. Fire, unpubl.).

rhIs4 pG75#KP6(*glr-1::gfp*) and pMH86 (H. Hutter, pers. comm.).

edIs6 pDP#MMUGF12(*unc-119::gfp*) and pRF4 (Maduro and Pilgrim 1995).

Complex array lines were produced by injection of a mixture of the linear reporter plasmid, a linear selectable marker plasmid, and an excess of *Pvu*II-fragmented genomic DNA from *C. elegans* as carrier. Plasmids were linearized with a unique blunt-cut enzyme in the vector backbone. Mass ratios (genomic DNA/plasmid DNA) were ~50–100:1, with the concentration of plasmid ~1–2 μg/ml.

ccEx6172 pSAK2(*myo-3::Ngfp–lacZ*) and pRF4, with excess genomic *C. elegans* DNA as carrier. Copy number of pSAK2 within the *ccEx6172* transgene was confirmed to be 1–2 (Southern blot analysis; data not shown).

ccEx6176 pNH#300(*egl-15::gfp*) and pMH86 with excess genomic *C. elegans* DNA as carrier.

Genetic screens

Screen for decreased expression of myo-3::gfp PD4251 animals carrying an integrated *myo-3::gfp* transgene expressing in bodywall and vulval muscles were mutagenized with EMS as described (Brenner 1974). Nonclonal F2 and F3 populations were observed under direct fluorescent illumination and animals with uniformly reduced GFP activity recovered for further analysis. We screened ~9000 F2 animals and identified four independent mutations with distinctly decreased GFP activity. One of these mutations was subsequently shown to be tightly linked to the original transgene and was not further characterized. The other three mutations (*cc566, cc567,* and *cc587*) were all recessive and failed to complement, defining the *tam-1* locus on chromosome V. We observed some differences in phenotype between alleles; *cc566* and *cc567* animals showed strongest reduction of *myo-3::gfp* activity in the center of the body, with lesser effects in head and tail regions. *cc587* showed a less dramatic reduction in GFP with no evident differences between head, body, and tail.

Screen for decreased expression of a lin-3 *transgene* Additional alleles of *tam-1* were identified as part of an independent screen for reversion of a Muv defect caused by an integrated high-copy *lin-3* transgene (*syIs1*). This screen yielded (1) several mutants corresponding to known components of the *lin-3*-signaling pathway (J. Liu and P.W. Sternberg, unpubl.) and (2) 15 alleles of *tam-1* (*sy270, sy272, sy273, sy342, sy366, sy372, sy394, sy395, sy398, sy399, sy401, sy402, sy410, sy411,* and *sy412*).

Genetic and molecular analysis of tam-1 Mapping of *tam-1* was carried out with allele *cc567* by standard methods. Three-factor mapping was done with *unc-46 dpy-11/tam-1*; *ccIs4251*. 16/16 Dpy-non-Unc picked up *tam-1*, whereas 10/10 Unc-non-Dpy did not pick up *tam-1*; also 9/9 Dpy-non-Tam picked up *unc-46*, indicating that *tam-1* is very close or to the left of *unc-46*. A genetic deficiency in this region, *nDf32* failed to complement *tam-1*. *unc-46* has thus been used as a linked marker in introducing *tam-1* into various genetic backgrounds. *unc-46* (without *tam-1*) has also been introduced into a variety of transgene-carrying strains and has shown no effect on transgene expression.

By use of the intensity of GFP fluorescence in adults carrying the *ccIs4251* transgene as an assay, all *tam-1* alleles tested (*cc567, cc566, cc587,* and *sy272*) were recessive and showed no evidence of maternal effect. This assay was used for all complementation and genetic rescue assays with *tam-1*.

Phenotypic rescue of *tam-1* mutants was assayed in transgenic lines derived from microinjection of DNAs into homozygous *tam-1* mutants (Mello and Fire 1995). Cosmid DNAs were injected at 10–20 μg/ml. To identify transgenic lines, plasmid pRF4, which contains the semidominant mutation *rol-6* (*su1006*), was coinjected at 100 μg/ml (Mello et al. 1991). For each cosmid mixture, several independent transgenic lines were recovered and scored for restoration of *gfp* expression to levels comparable with those seen in the parental strain PD4251. A positive pool of five cosmids was successively divided, leading to the identification of a single rescuing cosmid, F26G5. F26G5 was shown to rescue three alleles of *tam-1* (*cc566, cc567,* and *sy272*).

Protein localization To produce antisera recognizing TAM-1, two different regions of the cDNA (Pro594–Glu712 and Ala778–Ser856) were cloned into GST gene fusion vectors (pGEX-2T) (Smith and Johnson 1988) to produce fusion proteins of 39 and 35 kD. SDS-polyacrylamide gel slices containing purified fusion proteins were prepared for the immunization of mice. Polyclonal mouse sera were collected and used for immunohistochemistry as described (Miller and Shakes 1995). Freeze-cracked adult *C. elegans* were freeze substituted in methanol (–20°C, 4 min) and fixed with 4% formaldehyde (23°C, 30 min).

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Appendix 2

Interference with gene regulation in living sea urchin embryos: Transcription factor Knock Out (TKO), a genetically controlled vector for blockade of specific transcription factors

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Interference with gene regulation in living sea urchin embryos: Transcription factor Knock Out (TKO), a genetically controlled vector for blockade of specific transcription factors

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Contributed by Eric H. Davidson, October 1, 1998

ABSTRACT **"TKO" is an expression vector that knocks out the activity of a transcription factor *in vivo* under genetic control. We describe a successful test of this concept that used a sea urchin transcription factor of known function, P3A2, as the target. The TKO cassette employs modular cis-regulatory elements to express an encoded single-chain antibody that prevents the P3A2 protein from binding DNA *in vivo*. In normal development, one of the functions of the P3A2 transcription factor is to repress directly the expression of the *CyIIIa* cytoskeletal actin gene outside the aboral ectoderm of the embryo. Ectopic expression in oral ectoderm occurs if P3A2 sites are deleted from *CyIIIa* expression constructs, and we show here that introduction of an *αP3A2·TKO* expression cassette causes exactly the same ectopic oral expression of a coinjected wild-type *CyIIIa* construct. Furthermore, the *αP3A2·TKO* cassette derepresses the endogenous *CyIIIa* gene in the oral ectoderm and in the endoderm. *αP3A2·TKO* thus abrogates the function of the endogenous SpP3A2 transcription factor with respect to spatial repression of the *CyIIIa* gene. Widespread expression of *αP3A2·TKO* in the endoderm has the additional lethal effect of disrupting morphogenesis of the archenteron, revealing a previously unsuspected function of SpP3A2 in endoderm development. In principle, TKO technology could be utilized for spatially and temporally controlled blockade of any transcription factor in any biological system amenable to gene transfer.**

In this communication we describe a means of blocking a specific gene regulatory interaction in living sea urchin embryos. Sea urchins have a relatively simple process of embryogenesis, and an efficient and straightforward method of gene transfer has been developed, affording the opportunity to introduce expression constructs into thousands of eggs per day. Sea urchin eggs and embryos have thus emerged as a major experimental system for analysis of developmental cis-regulatory functions (1–3). Quantitative temporal and spatial patterns of reporter-gene expression can be conveniently assessed (e.g., refs. 3–5). Many relevant sea urchin transcription factors have been purified by using affinity chromatography after identification of their cis-regulatory target sites and subsequently have been cloned (6). Antisense oligonucleotides can be used to destroy maternal mRNAs encoding transcription factors; however (7), there has been no general or direct way to examine the function of specific transcription factors in sea urchin embryos by blocking their activity *in vivo*. This would afford the opportunity to compare the effects of canceling transcription factor activity with the effects of mutations of the relevant target sites in an expression vector (8). More generally, it would provide a means of determining downstream functions of the targeted factor.

Here we demonstrate the functional blockade of a transcription factor that had previously been shown to be responsible for spatial control of a developmentally regulated sea urchin embryo gene. This was accomplished by introducing into fertilized eggs an expression construct that encodes a single-chain antibody that binds to the factor and prevents it from forming complexes with its DNA target sites. We term this a "TKO" (*T*ranscription factor *K*nock *O*ut) vector.

The TKO vector described herein was designed to attack the SpP3A2 transcription factor (9), the initial member of a small family of transcription factors that now includes *Drosophila* erect wing (10), chicken IBR/F (11), and human NRF-1 (12). P3A2 was cloned and characterized (9, 13) after identification of two of its target sites in the cis-regulatory element of the *CyIIIa* cytoskeletal actin gene, which were found to be required for correct spatial expression of this gene. This gene is normally expressed only in aboral ectoderm lineages beginning early in development. *CyIIIa·CAT* expression constructs reproduce the aboral expression of the parent gene, but if either of the P3A2 sites in the wild-type construct is destroyed, expression spreads dramatically to the oral ectoderm (14). Similarly, if the endogenous P3A2 factor is titrated away from the *CyIIIa·CAT* expression construct by cointroduction of excess target-site, ectopic oral ectoderm expression is also observed (15). Our objectives in this work were threefold: first, to develop a TKO vector that would effectively sequester endogenous P3A2 transcription factor (*αP3A2·TKO*), the functionality of which could be assayed by determining its effects on spatial expression of *CyIIIa·CAT*; second, to determine whether we could affect the expression of the endogenous *CyIIIa* gene during embryonic development; and third, to look for any other phenotypes in *αP3A2·TKO* embryos that may indicate additional embryonic functions of the P3A2 transcription factor.

MATERIALS AND METHODS

DNA-Binding Inhibition Assays. P3A2 DNA-binding electrophoretic mobility-shift assays (EMSAs) were performed as previously described by Calzone *et al.* (9) using the P3A2 binding site (top strand: 5′-GATCTTTTCGGCTTCTGCG-CACACCCCACGCGCATGGGC-3′) and crude nuclear extracts. Inhibition assays were performed by incubating P3A2 DNA binding reactions with serial dilutions of supernatant from hybridoma-producing αP3A2 mAbs. The αP3A2 mAbs

Abbreviations: EMSA, electrophoretic mobility-shift assay; WMISH, whole-mount *in situ* hybridization; scFv, single-chain antibody; GFP, green fluorescent protein; H2b, histone 2b.

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were created in the Caltech Monoclonal Antibody Facility (16). Hybridoma ascites fluid and bacterial extracts containing the αP3A2 sites were similarly assayed. In these assays, inhibition of P3A2–DNA complex formation was estimated quantitatively as described in Fig. 1.

Production of the Single-Chain Anti-P3A2 Antibody. Hybridoma poly(A) RNA was purified by using the Stratagene RNA Isolation kit and Dynal (Great Neck, NY) oligo(dT) beads as described by the manufacturers. Heavy- and light-chain cDNAs were isolated from hybridoma cDNA libraries constructed by using the Stratagene Uni-Zap vector system. Subsequently, the variable heavy- and light-chain coding regions of these cDNAs were fused in-frame (separated by an encoded flexible Gly/Ser linker) into the pCANTAB 5 vector by using the expression module of the Pharmacia single-chain antibody (scFv) system.

Construction of TKO and Green Fluorescent Protein (GFP) Expression Cassettes. The TKO cassette was constructed in steps. First, the simian virus (SV)40 large T 3′ untranslated intron, transcriptional attenuation, and polyadenylation signals isolated from the pNL vector (17) were cloned into the *Eco*RI site of pBluescript II KS− [BSpoly(A) construct]. Second, the coding region of the αP3A2 scFv was recovered from the positive pCANTAB 5 phagemid by PCR, using either the Ab1 5′ primer to make the TKO expression cassette or the Ab3 5′ primer to make a missense control cassette. Primers were as follows: Ab1, 5′-GGGCGGCCGCCACCATGGCCACCGCCCCAAAGAAGAAGCGTAAGGCCCAGGTGAAACTGCAG-3′. The italicized base is deleted in primer Ab3 5′; otherwise, primers Ab1 5′ and Ab3 5′ are identical.

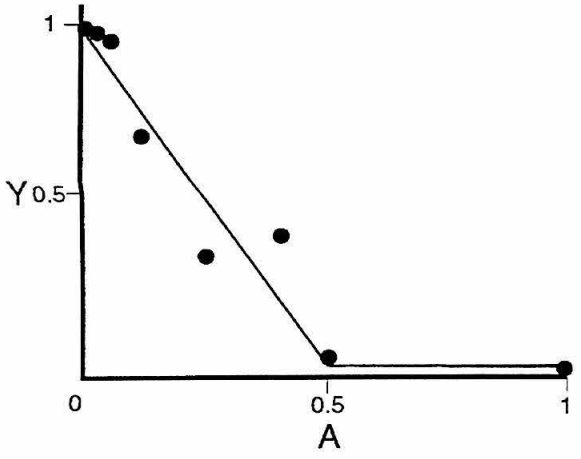

FIG. 1. Measurement of inhibitory activity of mAb with respect to specific P3A2–DNA complex formation. Relative occupancy of oligonucleotide probe (Y) is plotted against an antibody dilution series (A). The intercept, Y_0 (here $Y_0 = 0.78$ of total probe in the reaction) represents the probe occupancy in the absence of antibody ($A = 0$). The inhibitory activity is quantitatively estimated as the value of k_A, the slope; here, $k_A = 1.26 \times 10^{10}\ M^{-1}$. The data are fit to the function $Y = \alpha k_A A + Y_0$ (**Eq. 1**). Here Y is occupancy of oligonucleotide probe; i.e., where $PD_{(A)}$ dilution (antibody concentration was measured after purification of the antibody from the hybridoma ascites fluid) and D_0 is concentration of total probe in the reaction: $Y = PD_A/D_0$. In **Eq. 1**, $\alpha = -2P_0$, where P_0 is the molar quantity of P3A2 transcription factor in the reaction; in these experiments, P_0 ranged from 2 to 5×10^{-11} M. k_A is defined as the equilibrium constant for the reaction of a mAb molecule with two P3A2 molecules, i.e., $k_A = AP_2/A \cdot P_0^2$. Eq. (1) follows from the assumption that all P3A2 molecules will be in complex with either the antibody or the DNA probe and that the P3A2–DNA reaction is bimolecular, i.e., $PD_{(A)} = k_D P_{(A)} \cdot D_{(A)}$, where $P_{(A)}$ and $D_{(A)}$ are the molar concentrations of the free P3A2 protein and the DNA probe at given antibody dilutions.

Sequences encoding a nuclear localization site following the ATG site: Ab-reverse, 5′-GGGACTAGTCTTGTCGTCGTCGTCCTTGTAGTCCACCGGCGCACCTGCGGC-3′. Standard PCR was carried out in 100-μl volumes containing 50 pmol of each primer, 1× Deep Vent buffer (New England Biolabs), 2 μmol of dNTPs, ≈10 ng of target sequence, and sterile H_2O. Reactions were heated to 94°C for 2 min, 5 units of Deep Vent polymerase was added (New England Biolabs), and the mixture was cycled 25 times (30 sec, 94°C; 30 sec, 60°C; 60 sec, 72°C) in a Perkin–Elmer 9600 thermocycler. The PCR products were digested with *Not*I, purified, and ligated into the BSpoly(A)1 vector. The final TKO and missense cassettes were made by ligating either the cis-regulatory element (18) of the SpH2b early histone gene into the *Sac*II site or the SpHE hatching enzyme gene cis-regulatory element (a gift from L. Angerer, University of Rochester, Rochester, NY; see ref. 19) into the *Sac*I site of the respective TKO and missense constructs. The histone gene regulatory sequences were amplified from the SpH2b clone (18) with the following oligonucleotide primers: H2b5, 5′-CCCGCGGCCGCGGTCTCAAAATATGATTGGCAGCTTAATTTGG-3′ and H2b3, 5′-CCCCCGCGGGATGATTGTGATTCTCACGAATGC-3′. The hatching enzyme gene regulatory sequence was amplified from the parent SpHE-pNL clone by using HE5: 5′-CCCCCGCGGAAGCTTGTTTTGATTGGTTTGTTTGG-3′ and HE3: 5′-CCCCCGCGGGATGATAAGAAGTGATAATGATTTTC-3′. The pCANTAB5 anti-P3A2 scFv and TKO inserts were sequenced in an Applied Biosystems 373 sequencer. TKO and αP3A2·TKOmis (missense) plasmids were linearized with *Cla*I for microinjection. A pHE/GFP expression vector was constructed by amplifying the SpHE regulatory element with primers HESI5, 5′-CCCGAGCTCAAGCTTGTTTTGATTGGTTTGTTTGG-3′ and HE3, 5′-CCCGAGCTCGATGATAAGAAGTGATAATGATTTTC-3′ and ligating the product into the GFP expression vector pGL3-Basic (5). The resulting plasmid was linearized with *Kpn*I for microinjection.

Microinjection, Whole-Mount *in Situ* Hybridization, and Fluorescence Microscopy. Microinjection and culture of *Strongylocentrotus purpuratus* embryos were performed essentially as described (14). However, when multiple constructs were coinjected, we maintained the total amount of DNA injected at a constant level by proportionately lowering the amount of carrier DNA. Detection of endogenous *CyIIIa* expression by whole-mount *in situ* hybridization (WMISH) was carried out following the protocol of Ransick *et al.* (24) with the following modifications: The fixation step was lengthened to 20 min and the probe concentration was raised to 0.4 ng/μl. The *CyIIIa* antisense probe was generated by *in vitro* transcription from the T7 promoter of the *CyIIIa–Hga*I vector that had been digested with *Pvu*II. This generates a digoxigenin-labeled probe containing 147 bases of the *CyIIIa*, 3′ untranslated region, and an 873-bp tail of pBluescript II SK− (procedure of David G.-W.Wang, personal communication). Fluorescence microscopy, sorting, and WMISH of GFP-injected embryos were performed as described (5).

RESULTS

αP3A2 mAb and *αP3A2·TKO* Construct. The TKO expression construct was designed to express, translate, and transport an anti-P3A2 reagent into the nucleus so as to inhibit P3A2 from carrying out its developmental regulatory functions *in vivo*. A set of αP3A2 mAbs was generated against the full-length P3A2 protein. These were screened for their ability to inhibit this protein from binding to its target DNA sequence by adding them to gel-shift reactions at increasing dilutions. Inhibitory activity was measured quantitatively as described in Fig. 1. Inhibition constants were determined for 12 different hybridoma subclones. mAb 7B12/2E7 displayed the strongest inhibitory effects on P3A2 DNA binding and was chosen as the

basis for the *αP3A2·TKO* vector. The same mAb was used for an extensive series of measurements of P3A2 prevalence throughout embryological development (16). The specificity of this antibody was confirmed by the observation that it reacted with only a single molecular species of the known size of the P3A2 transcription factor in total nuclear extracts from all stages of development as well as in total egg lysate (Fig. 1 of ref. 16).

To build the *αP3A2·TKO* vector, the mRNA was extracted from 7B12/2E7 hybridoma cells, and by using the universal IgG primers amplified DNAs encoding the heavy-chain and light-chain V-regions were cloned in-frame into the Pharmacia pCANTAB vector surrounding a flexible Gly/Ser linker. Thus, a construct encoding a single-chain antibody was produced. Gel-shift inhibition assays were repeated to test the inhibitory activity of the αP3A2 scFv, which was extracted for this purpose from bacteria expressing the pCANTAB vector. Its activity was found to be quantitatively equivalent to that of the starting mAb. As described in *Materials and Methods*, the αP3A2 scFv was then incorporated into an expression cassette, the organization of which is summarized in Fig. 2. In addition to the scFv itself, the major features of the *αP3A2·TKO* construct are: (*i*) a nuclear localization site intended to increase the concentration of the scFv in the nucleus relative to that of the endogenous nuclear P3A2 factor; (*ii*) simian virus (SV)40 intron, 3′ trailer, termination, and poly(A) addition sites, features that are conventionally used in most sea urchin gene-transfer vectors; and (*iii*) a cis-regulatory control element. This last design feature, in principle, permits expression of the TKO vector to be targeted to any domain of the embryo for which a cis-regulatory element is available. However, in the present experiments we utilized instead ubiquitously, or nearly ubiquitously, active cis-regulatory elements derived from a histone H2b gene (18) and from a hatching enzyme gene (19). Stable incorporation of exogenous DNA in sea urchin embryo blastomeres is mosaic, although entirely random with respect to lineage (20), and therefore embryos are obtained that have incorporated the exogenous DNA into every possible spatial domain. In some of the experiments that follow, the *αP3A2·TKO* vector was coinjected with a GFP reporter under control of the same cis-regulatory element, so that the particular cells incorporating the *αP3A2·TKO* construct could be identified visually by GFP expression following the procedures of Arnone *et al.* (5). Thus, we could examine embryos in which the construct was present in endoderm, aboral ectoderm, or oral ectoderm, as desired.

***αP3A2·TKO* Effects on Spatial Expression of a Wild-Type *CyIIIa·CAT* Construct.** When *CyIIIa·CAT* constructs lacking the two known P3A2 target sites are injected into eggs, ectopic oral ectoderm expression is observed in an average of 34% of stained embryos (14). Because this is statistically equivalent to the percentage of embryos that incorporate exogenous DNA into oral ectoderm founder cells, it follows that essentially all oral ectoderm cells will express the *CyIIIa·CAT* construct if interactions with the P3A2 repressor are obviated by deletion. In the *CyIIIa·CAT* experiments summarized in Table 1, *αP3A2·TKO* or a missense frame shift control differing from *αP3A2·TKO* by deletion of only 1 bp were coinjected with wild-type *CyIIIa·CAT* (14). Wild-type *CyIIIa·CAT* and *ΔP3A2·HF*, the *CyIIIa·CAT* construct lacking the two P3A2 sites, were injected at the same time as the TKO constructs and performed as reported (14). When coinjected with wild-type *CyIIIa·CAT*, the missense *αP3A2·TKO* had no effect (2% oral expression). However, a remarkable result was obtained when wild-type *CyIIIa·CAT* was coinjected with *αP3A2·TKO*: 26% of stained embryos now displayed oral ectoderm expression, as illustrated in the example reproduced in Fig. 3 *c* and *d*. The 26% value could represent a slight underestimate of *αP3A2·TKO* effects or *CyIIIa·CAT* expression because in these experiments only morphologically normal stained embryos were counted, and as we discuss below, a significant fraction of embryos receiving *αP3A2·TKO* develop abnormally. In any case, the results are those predicted if the *αP3A2·TKO* vector effectively sequesters the endogenous P3A2 factor, thus preventing its interaction with the *CyIIIa·CAT* regulatory target sites. That is, as Table 1 shows, the fraction of ectopically expressing embryos is about the same as when P3A2–*CyIIIa·CAT* interactions are instead prevented by deletion of these target sites.

We note here that coinjection of the anti-P3A2 antibody protein into eggs together with *CyIIIa·CAT* also led to ectopic oral expression of the *CyIIIa·CAT* reporter (C. Kirchhamer and E.H.D., unpublished results). These effects were not observed when an irrelevant antibody was injected. However, this approach can be applied only to early embryos; furthermore, the αP3A2 and the control IgG caused lethal arrest of development in an unacceptably large fraction of embryos.

***αP3A2·TKO* Effects on Spatial Expression of the Endogenous *CyIIIa* Gene.** The foregoing results confirm the role of the P3A2 transcription factor as a spatial repressor of *CyIIIa·CAT* expression and raise the question whether the endogenous gene could be similarly affected by *αP3A2·TKO*. This was not obvious *a priori*, because we had found earlier that the stability of endogenous *CyIIIa* regulatory complexes exceeds that of the equivalent *CyIIIa·CAT* complexes: thus, *in vivo* competition with excess target sites for a number of *CyIIIa* factors stoichiometrically reduced expression of exogenous *CyIIIa·CAT*, whereas expression of the endogenous *CyIIIa* gene was unaffected in the same embryos (22).

The normal aboral ectoderm expression of the endogenous *CyIIIa* gene is displayed by WMISH in Fig. 3 *a* and *b*. When eggs were injected with *αP3A2·TKO* and endogenous *CyIIIa* expression was similarly monitored, ectopic expression of the

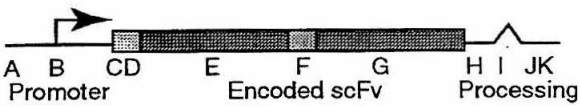

FIG. 2. The TKO expression cassette. Histone 2b or hatching enzyme cis-regulatory elements (A) employed in the cassette are modular and can easily be exchanged with any other regulatory sequence. The start of transcription (B) is set to be at least 20 bp upstream of a canonical Kozak ATG (C) and a nuclear localization sequence (D) that are best fit to sea urchin initiators and codon usage. The heavy- (E) and light-chain (G) variable regions of the anti-P3A2 scFv are separated by an encoded flexible Gly/Ser linker (F). To process, stabilize, and terminate the TKO transcripts, we inserted the SV40 large T 3′ intron (I), polyadenylation (J), and transcriptional termination (K) sequences after the stop codon (H).

Table 1. Effect of *αP3A2·TKO* and control constructs on *CyIIIa·CAT* and *CyIIIa* expression

Construct	48-hr embryo no. tested*	Ectopic mRNA in oral ectoderm, %		mRNA in aboral ectoderm, %	
		CyIIIa	*CAT*	*CyIIIa*	*CAT*
CyIIIa·CAT† (wild type)	531	—	3.2	—	96
ΔP3A2·HF†	192	—	34	—	95
αP3A2·TKO	108	—	26	—	83
αP3A2·TKOmis	184	—	2	—	99
αP3A2·TKO	70	27	—	100	—
αP3A2·TKOmis	35	8	—	100	—

*Data are reproduced for interpretably stained embryos (60–901 of all those injected in ref. 14 and this study).

†Data reproduced from Kirchhamer and Davidson (14) for comparison.

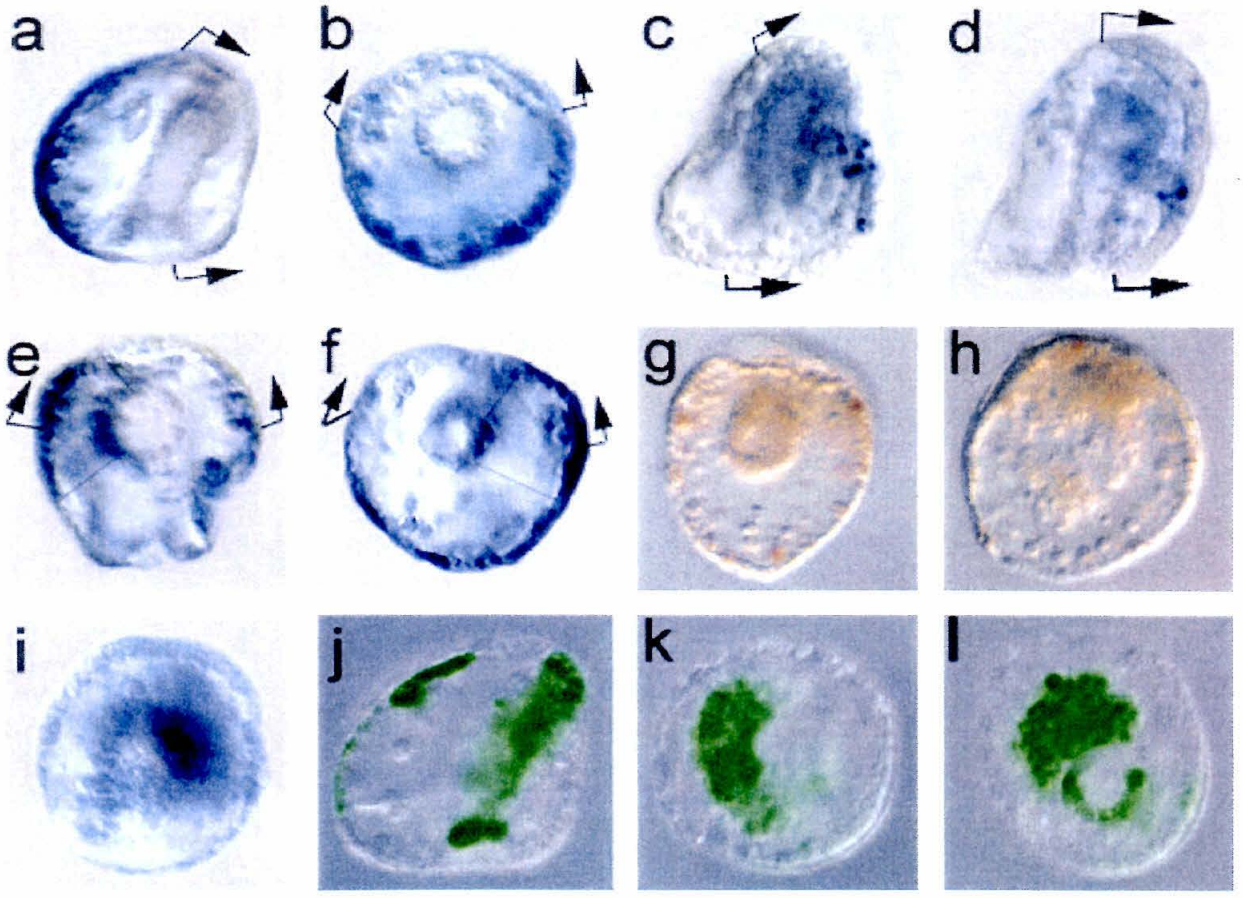

FIG. 3. Effects of *αP3A2·TKO* on expression of *CyIIIa·CAT*, endogenous *CyIIIa*, and the lethal embryonic phenotype. In *a–f*, the H2b cis-regulatory element was used to drive expression of the injected TKO constructs, and in *g–l*, the hatching enzyme cis-regulatory element was used (see *Materials and Methods*). (*a*, *b*) Endogenous *CyIIIa* expression monitored by WMISH using an antisense *CyIIIa* probe (see *Materials and Methods*). (*a*) Embryo viewed laterally, oral side to the right; here and in following panels the arrows bracket the oral ectoderm where neither *CyIIIa* nor wild-type *CyIIIa·CAT* expression occurs. (*b*) Anal view, oral side to the top. (*c*, *d*) *CyIIIa·CAT* coinjected with *αP3A2·TKO*, driven by the H2b cis-regulatory element, lateral views. Clones of cells expressing CAT mRNA in the oral ectoderm are displayed by using WMISH. As observed when the P3A2 sites are deleted (14), these injections produce embryos displaying normal patterns of aboral ectoderm expression (i.e., embryos in which the exogenous DNA resides in the aboral ectoderm); embryos displaying both normal and ectopic oral ectoderm expression; and embryos displaying oral expression only (i.e., the exogenous DNA is only in oral ectoderm lineages); only the latter are illustrated here. (*e*, *f*) Effects of *αP3A2·TKO* on endogenous *CyIIIa* gene expression, monitored as in *a* and *b*. Both embryos are shown from the blastopore, and both display ectopic oral staining as well as staining in clones of archenteron cells in addition to aboral ectoderm expression. (*g*) Pluteus-stage (72-hr) embryo viewed from anal side. This embryo bears the *αP3A2·TKOmis* construct and is normal in morphology. (*h*) Gastrulation-defective phenotype at 72 hr caused by injection of the *αP3A2·TKO* construct. (*i*) Gastrulation-defective embryo at 48 hr viewed from blastopore, in which *Endo16* transcripts are displayed by using WMISH. (*j*) Normal 48-hr embryo viewed laterally and bearing GFP-expressing clones of exogenous DNA in aboral ectoderm and archenteron. These clones also include the *αP3A2·TKOmis* control construct. (*k*, *l*) Gastrulation-defective embryos as in *j*, but bearing *αP3A2·TKO* construct present in large endodermal clones.

endogenous gene was observed. As summarized in Table 1, 27% of these embryos display clear expression in clones of oral ectoderm, and frequently in limited clones of gut cells as well. Examples are reproduced in Fig. 3 *e* and *f*. Three embryos that displayed ectopic expression in the missense control were also found (Table 1). Because the endogenous *CyIIIa* gene is never expressed erroneously, the single base mutation that distinguishes *αP3A2·TKO*mis from *αP3A2·TKO* might not have entirely prevented synthesis of a functional TKO product. For example, a partially functional protein might have been generated by use of a downstream ATG. In any case, the percentage of ectopic oral expression observed in the *αP3A2·TKO* sample is very similar to that seen with either the mutated *CyIIIa* reporter lacking P3A2 sites (14) or in the coinjections of *αP3A2·TKO* and wild-type *CyIIIa* reporters (Table 1). This is as expected, because ectopic oral expression depends on clonal incorporation of the *αP3A2·TKO* construct in oral ectoderm cells in all three experiments.

A Specific *αP3A2·TKO* Embryonic Phenotype. Embryos developing from eggs injected with *αP3A2·TKO* display a specific lethal phenotype. This occurs in a relatively high fraction of embryos, 44% in the experiment tabulated in Table 2, although in other experiments somewhat more modest frequencies of 20–30% were recorded. The *αP3A2·TKOmis* control resulted in only 5% abnormal embryos (Table 2). This phenotype is best described as a failure to form a complete archenteron, caused by a disorganization of the endoderm. Fig. 3*g* shows a normal 72-hr prism-stage embryo that developed from an egg that had been injected with the missense control construct, whereas the embryo in Fig. 3*h* displays the

Table 2. *αP3A2·TKO* effect on embryo development

Construct	Embryos, no. tested	Gastrulation-defective, %
αP3A2·TKO	419	44
αP3A2·TKOmis	345	5

αP3A2·TKO phenotype. This embryo has not completed gastrulation. Its endoderm consists of a pile of nonadherent cells, and the form of the embryo is round rather than triangular. The secondary mesoderm appears unaffected, however. Such embryos appear to develop normally up to gastrulation. The implication is that P3A2 transcription factor is necessary for morphogenesis of the archenteron, a previously unknown function.

To further investigate the role of the P3A2 transcription factor in archenteron formation, we performed a series of coinjection experiments making use of GFP as a marker to determine location of the *αP3A2·TKO* construct in the experimental embryos. GFP expression constructs can be used to determine the mosaic expression domain of a coinjected construct if both constructs are driven by the same cis-regulatory elements, because shortly after injection the exogenous DNAs are all ligated together into a concatenate and are then incorporated into the same blastomeres (5). In these experiments, the hatching enzyme cis-regulatory element was used to drive both the GFP and *αP3A2·TKO* constructs. Results are listed in Table 3. Significantly, all of the embryos bearing *αP3A2·TKO* that failed to gastrulate express the GFP marker in the endoderm (89 of 89), and less than 10% of the set of embryos in which GFP is expressed in the endoderm develop normally. All of those that do are distinguished by small GFP clone sizes (not shown). On the other hand, most of the 48% of the embryos injected with the missense control that contained endodermal clones developed normally, even though these clones were often quite large. An example is shown in Fig. 3*j*. Embryos bearing *αP3A2·TKO* that failed to gastrulate and that contained the exogenous DNA in the endoderm are illustrated in Fig. 3 *k* and *l*. The disorganized endoderm cells in these embryos retain their endodermal state of specification; that is, they continue to express the *Endo16* marker (2, 23, 24), as shown, for example, in Fig. 3*i*. A few embryos in the missense control experiment also showed gastrulation defects (Table 3), and all of these contained large endodermal clones. This result again supports a weak expression of an *αP3A2·TKO* activity because of override of the frameshift, as considered above.

To explore further the relation between location of clonal incorporation and occurrence of the gastrulation phenotype, we carried out the experiment summarized in Table 4. Embryos bearing *αP3A2·TKO* plus GFP constructs were sorted according to the locus of incorporation of the expression constructs, and *CyIIIa* expression was then monitored by WMISH in the sorted samples. Table 4 shows that the locus of incorporation correlates perfectly with the effect of *αP3A2·TKO* on *CyIIIa* expression. All embryos selected for large endoderm clones again were gastrulation-defective (19 of 19), whereas 9 of 10 embryos that contained large oral-ectoderm clones displayed ectopic expression of *CyIIIa* in the oral ectoderm. Conversely, all embryos bearing large aboral ectoderm clones displayed normal *CyIIIa* expression.

Table 3. Expression of GFP in *αP3A2·TKO* and *α3PA2·TKOmis* embryos

Construct	Phenotype	Embryos, no. tested	Endomeso-derm, %	Ectoderm, %
αP3A2·TKO	Normal	77	9	95
αP3A2·TKO	GD	89	100	73
αP3A2·TKOmis	Normal	186	48	78
αP3A2·TKOmis	GD	15	100	73

GD, gastrulation defective.

Table 4. *CyIIIa* transcripts in presorted embryos bearing GFP and TKO (*αP3A2·TKO*) expression constructs

Phenotype	GFP sorted	Endomesoderm	Oral	Aboral
GD	Endoderm	19/19	ND	ND
Normal	Oral	0	9/10	10/10
Normal	Aboral	0	0	9/9

GD, gastrulation-defective; ND, not done. Oral and aboral ectodermal domains are difficult to distinguish in gastrulation-defective embryos, because of their abnormally rounded form (see Fig. 3*h*, for example).

It may be concluded from these experiments that the function of P3A2 in archenteron morphogenesis is cell-autonomous, i.e., that the factor is needed in the archenteron cells themselves. Similarly, we confirm that the function of P3A2 in oral ectoderm as a spatial repressor of *CyIIIa* transcription is also autonomous, as proposed earlier (14).

DISCUSSION

We describe here the use of a genetic expression vector that abrogates the activity of a transcription factor in a living embryo. Three main results were obtained. First, the intracellular efficacy of a scFv for this purpose was established by demonstration of the same ectopic expression of the *CyIIIa·CAT* vector as is caused by deletion of the target sites for this transcription factor. Second, we confirmed that the P3A2 factor indeed serves as a spatial repressor for the *CyIIIa* gene and extend this observation from *CyIIIa·CAT* expression constructs to the endogenous *CyIIIa* gene. Third, we discovered a hitherto unknown function of P3A2 in archenteron morphogenesis. Both this function and the effects of the P3A2 factor on *CyIIIa* expression are cell autonomous.

It is striking that *CyIIIa·CAT* and *CyIIIa* gene expression are so efficiently expanded to the oral ectoderm in embryos bearing *αP3A2·TKO* in oral ectoderm clones. P3A2 is initially a relatively prevalent maternal factor, present in about 2×10^6 molecules per embryo (16) and is later zygotically transcribed; there are a few thousand to a few hundred molecules per nucleus throughout development (16, 25). The affinity of the scFv encoded by the *αP3A2·TKO* vector for the P3A2 factor is significantly higher than that of the factor for its DNA target site (9, 13) as calculated from experiments such as that shown in Fig. 1. Thus, the scFv apparently acts as a very effective intracellular sequestering agent. Nor is any nonspecific early embryonic lethality observed, in contrast to the general deleterious effect of injecting the mAb *per se* into eggs (unpublished results). Furthermore, use of a genetic vehicle for producing the αP3A2 scFv ensures that it will continue to function through development (depending of course on the cis-regulatory element driving its expression), which is never reliably the case for either protein or mRNA introduced directly into the egg. The results shown in this paper confirm that *αP3A2·TKO* continues to function through embryogenesis (Fig. 3).

An unexpected aspect of the spatial derepression of the endogenous *CyIIIa* gene caused by introduction of *αP3A2·TKO* is that ectopic expression was observed not only in oral ectoderm but also in gut (Fig. 3 *e* and *f*). Recent studies (ref. 14, and earlier observations reviewed therein) proved that the two P3A2 sites deleted in the experiments of Kirchhamer and Davidson (14) are required to prevent ectopic oral-ectoderm expression of *CyIIIa·CAT* constructs, but no ectopic gut expression was observed in these experiments. However, there are additional potential P3A2 sites in the *CyIIIa* cis-regulatory element, and these may be responsible for controlling expression in gut.

A new role for P3A2 in archenteron formation was revealed when we examined the postgastrular lethal phenotype produced by *αP3A2·TKO* injection into fertilized eggs. This effect occurs only when the construct is present in large clones of archenteron cells. These cells fail to adhere to one another so that the archenteron disintegrates into a wholly or partially disorganized pile of endoderm cells. Yet these cells retain their state of specification, as monitored by expression of an endoderm-specific gene, *Endo16*. The implication is that genes encoding some cell surface proteins are controlled by P3A2 either directly or indirectly. If directly, it is possible that P3A2 acts positively in this context, although its function is clearly negative in regulation of the *CyIIIa* gene.

There are many obvious possible extensions of the TKO technology described here, both in research and potentially for gene therapy (26). We are now extending the TKO approach to several other sea urchin embryo transcription factors. This may become a general method for examination of trans-regulatory functions. One powerful potential advantage of the TKO method is the possibility of exactly controlling the time and place of TKO expression. This depends directly on the cis-regulatory system employed to drive the transcription of the TKO vector. Thus, for example, TKO vectors could be built and inserted into the mouse genome that would blockade given transcription factors only in given cell types at given stages of development. This could provide a decisive advantage given that transcription factors are often utilized at many stages of the life cycle and used for many diverse developmental purposes.

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