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sli-3 Negatively Regulates the LET-23/Epidermal Growth Factor Receptor-Mediated Vulval Induction Pathway in *Caenorhabditis elegans*

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ABSTRACT

The LIN-3–LET-23-mediated inductive signaling pathway plays a major role during vulval development in *C. elegans*. Studies on the components of this pathway have revealed positive as well as negative regulators that function to modulate the strength and specificity of the signal transduction cascade. We have carried out genetic screens to identify new regulators of this pathway by screening for suppressors of *lin-3* vulvaless phenotype. The screens recovered three loci including alleles of *gap-1* and a new gene represented by *sli-3*. Our genetic epistasis experiments suggest that *sli-3* functions either downstream or in parallel to nuclear factors *lin-1* and *sur-2*. *sli-3* synergistically interacts with the previously identified negative regulators of the *let-23* signaling pathway and causes excessive cell proliferation. However, in the absence of any other mutation *sli-3* mutant animals display wild-type vulval induction and morphology. We propose that *sli-3* functions as a negative regulator of vulval induction and defines a branch of the inductive signaling pathway. We provide evidence that *sli-3* interacts with the EGF signaling pathway components during vulval induction but not during viability and ovulation processes. Thus, *sli-3* helps define specificity of the EGF signaling to induce the vulva.

INDUCTION by intercellular signals mediated by growth factors play important roles in cell proliferation and fate specification. The intracellular signaling pathway mediated by epidermal growth factor (EGF) and its receptor (EGFR) has been extensively studied in many systems, and much is known about its components and how they are activated or inactivated (Moghal and Sternberg 2003b; Sundaram 2005). However, a signaling pathway is not a simply binary device, being either active or inactive. Once a pathway is activated, it can have many levels of activity. How these activities are regulated is not fully understood.

In *Caenorhabditis elegans*, the LIN-3–LET-23-mediated inductive signaling pathway is known to play multiple roles during development (Moghal and Sternberg 2003b). LIN-3 is a member of the EGF family that binds with the LET-23/EGFR to activate downstream pathway components including LET-60/Ras. Genetic analyses of the EGF signaling in *C. elegans* have revealed at least five different roles in regulating vulval formation, viability, ovulation, male spicule development, and posterior ectodermal P12 cell fate specification (Moghal and Sternberg 2003b). During vulval development and P12 fate specification activated LET-23 receptor transduces a signal through a conserved set of factors that includes LET-60/Ras, LIN-45/Raf, and MPK-1/MAP kinase. However, the ovulation process is mediated by a Ras-independent pathway and involves calcium signaling, which is regulated by inositol trisphosphate (IP_3) and its receptor ITR-1 (Clandinin *et al.* 1998). Thus, different outcomes of the signaling depend upon tissue-specific effectors.

The molecular genetic studies of EGFR signaling during vulval development have revealed both positive and negative components that function to regulate the strength and specificity of the signal transduction cascade. The vulva is formed by the progeny of three (P5.p, P6.p, and P7.p) of six (P*n*.p, $n = 3$–8) equipotential vulval precursor cells (VPCs) that are induced by the LIN-3/EGF produced by the gonadal anchor cell. Once induced, these three VPCs acquire 1° and 2° cell fates and undergo three rounds of cell divisions. Vulval progeny differentiate to form vulval tissue in adult animals.

Genetic analysis of the *C. elegans* vulva has revealed that in addition to LET-23-mediated inductive signaling two additional pathways, lateral signaling mediated by LIN-12/Notch and Wnt signaling mediated by BAR-1/β-Catenin, also participate in vulval development (Eisenmann 2005; Greenwald 2005; Sternberg 2005).

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Thus, vulval formation serves as a powerful system to investigate the underlying mechanisms regulating interactions between three evolutionarily conserved signaling pathways. Such studies have begun to reveal the components of these signaling pathways and their regulators, as well as target genes conferring specific developmental outcomes [*e.g.*, *lin-39/Hox*, *dpy-22/TRAP230*, and *lag-1/Su(H)/CBF1*] (Christensen *et al.* 1996; Eisenmann *et al.* 1998; Moghal and Sternberg 2003a). To identify additional regulators of the EGF signaling pathway, we carried out a genetic screen to isolate suppressors of the *lin-3(rf)* Vulvaless (Vul) phenotype. Here, we report the isolation and characterizations of three mutations, one of which represents a new locus *sli-3*. The genetic analysis of *sli-3* has revealed its function as a negative regulator of the EGF signaling pathway in vulval cells. Our epistasis experiments show that *sli-3* functions either downstream or in parallel to transcriptional regulators *lin-1* and *sur-2*. We also demonstrate that *sli-3* specifically participates in the vulval function of EGF signaling but not in other developmental processes, *i.e.*, viability, ovulation, and P12 cell fate specification. Furthermore, we find that *sli-3* does not genetically interact with *lin-12/Notch* and Wnt pathway components *bar-1*/β-*catenin* and *pry-1/axin*. Taken together, our findings establish *sli-3* as a tissue-specific regulator of the EGF signaling that helps establish proper signaling intensity during vulval development.

MATERIALS AND METHODS

General methods: Worms were grown according to published methods (Brenner 1974). All experiments were performed at room temperature (20°) unless otherwise noted. Cell and tissue anatomy was observed under Nomarski DIC optics as described by Sulston and Horvitz (1977). Standard cellular and genetic nomenclature is as defined by Sulston and Horvitz (1977) and Horvitz *et al.* (1979).

Vulval induction was observed by scoring the number of VPCs that adopt vulval fates during the L4 stage. In wild-type animals (N2) vulval induction is three (one for each P5.p, P6.p, and P7.p). However, mutant animals have variable vulval induction (zero to six range). The vulval lineage was determined by direct observations of the cell-division patterns in animals between mid-L3 and early-L4 stages.

Strains and construction strategies: The wild-type N2 and standard mutant strains are from Brenner (1974) and the *Caenorhabditis* Genetics Center. The mutants strains used in this study are as follows (references are given where appropriate):

LGI: *pry-1(mu38)* (Maloof *et al.* 1999), *sur-2(ku9)* (Singh and Han 1995), *unc-13(e51)* (Brenner 1974), *unc-101(sy108)* (Lee *et al.* 1994), and *dpy-5(e61)* (Brenner 1974).

LGII: *dpy-10(e128)*, *unc-4(e120)*, *rol-1(e91)*, *rol-6(e187)* (Brenner 1974); *let-23(sy15)*, *let-23(sy97)* (Aroian and Sternberg 1991); *vab-9(e1744)*, *let-239(mn93)* (Sigurdson *et al.* 1984); *mnC1[dpy-10(e128) unc-52(e444)]* (Herman 1978); *mnDf29*, *mnDf44*, *mnDf46*, *mnDf58*, *mnDf61*, *mnDf62*, *mnDf67*, *mnDf68*, *mnDf69*, *mnDf85*, *mnDf89*, *mnDf90*, *mnDf106* (all deficiencies are from Sigurdson *et al.* 1984); and *mnDp34* (Herman *et al.* 1979).

LGIII: *dpy-18(e364)* (Brenner 1974), *lin-39(n709)* (Garriga *et al.* 1993), and *lin-12(n137n460)* (Greenwald *et al.* 1983).

LGIV: *ark-1(sy187)* (Hopper *et al.* 2000); *let-59(s49)*, *let-312(s1234)* (Clark *et al.* 1988); *itr-1(sy290)* (Clandinin *et al.* 1998); *dpy-20(e1282)*, *unc-22(s7)*, *unc-24(e138)* (Moerman and Baillie 1979); *let-60(n2034)*, *unc-31(e169)* (Beitel *et al.* 1990); *lin-45(sy96)* (Han *et al.* 1993); *lin-3(n378)*, *lin-3(n1058)*, *lin-3(n1059)*, *nT1[unc(n754dm) let]* = *DnT1* (Ferguson and Horvitz 1985); and *sDf63*, *sDf67* (Clark and Baillie 1992).

LGV: *dpy-11(e224)*, *him-5(e1490)*, *unc-34(e315)* (Hodgkin *et al.* 1979); and *lin-25(e1446)*, *nT1[unc(n754dm) let](IV, V)* (Ferguson and Horvitz 1985).

LGX: *bar-1(ga80)* (Eisenmann *et al.* 1998); *dpy-6(e14)*, *dpy-8(e120)*, *lon-2(e678)*, *unc-1(e719)*, *unc-2(e55)*, *unc-6(e78)* (Brenner 1974); *unc-97(su110)* (Zengel and Epstein 1980); *gap-1(n1691)* (Hajnal *et al.* 1997); *sli-1(sy143)* (Jongeward *et al.* 1995); and *mnDp31* (Herman *et al.* 1979).

To screen for the suppressors of *lin-3* Vul phenotype, we constructed a strain PS1031 [*let-312(s1234) lin-3(n378) unc-22(s7)/unc-24(e138) lin-3(n1059) dpy-20(e1282)*] that carries two *lin-3* hypomorphic alleles, *n378* and *n1059*. PS1031 was constructed as follows: *let-59 unc-22/nT1; +/nT1* hermaphrodites were mated with N2 males and the F_1 males were mated with *let-312 lin-3(n378)/DnT1; +/DnT1* hermaphrodites. Individual F_1 non-Unc hermaphrodites were picked and *let-312 lin-3(n378)/let-59 unc-22* progeny were identified on the basis of their segregation of both early and late larval lethals. The Unc progeny of these worms were crossed with N2 males, and F_1 males were mated with *unc-24 lin-3(n1059) dpy-20/DnT1; +/DnT1* hermaphrodites. Non-Unc cross-progeny were picked at L4 and those that became Vul adults were *let-312 lin-3(n378) unc-22/unc-24 lin-3(n1059) dpy-20*. In this strain vulval induction is severely defective (1% of wild type, see Table 1; also see Ferguson and Horvitz 1985) and animals exhibit fully penetrant egg-laying defective phenotype.

To construct a *sli-3; lin-3(n1058)* strain, *unc-24 n1058/DnT1; +/DnT1* hermaphrodites were crossed with N2 males. The non-Unc F_1 males (*unc-24 n1058/+*) were mated with *dpy-10 sy341* hermaphrodites and *dpy-10 sy341; n1058 dpy-20* animals were obtained in two generations.

To construct a partial triploid strain carrying extra copies of *sli-3*, we used a free duplication *mnDp34*. For this, *mnC1/unc-4 unc-52; mnDp34* hermaphrodites were mated with *unc-4 sy341/+; n378* males and F_1 worms were individually cloned. In the next generation non-Unc (*unc-4*) non-Dpy Egl worms were picked from a clone that segregated Unc (*unc-4* non-*unc-52*) animals to establish a line of *unc-4 sy341/mnC1; n378; mnDp34* animals. The presence of *mnDp34* was confirmed by segregation of the Dpy animals.

For deficiency mapping of *sy341*, we constructed *sy341/Df* strains by mating *dpy-10 sy341; n378* hermaphrodites with *Df/mnC1; n378* males. In the F_1 generation non-Dpy worms were individually picked to establish clonal populations.

Mutagenesis: Worms were mutagenized by ethyl methanesulfonate (EMS) (Brenner 1974). To isolate the suppressors of the *lin-3* Vul phenotype, we used strain PS1031 (see above) and screened ~30,000 haploid genomes. Single non-Egl F_2 worms from each P0 plate were individually cloned. For those plates that carried <10 progeny, worms were allowed to grow two additional generations before picking a putative Egl suppressor. We scored vulval induction of the progeny to confirm the phenotype and obtained five true breeding non-Egl lines.

To screen for *sli-4* revertants, we mutagenized animals carrying the *sy330* allele in a PS1031 background. Single Egl hermaphrodites were picked in F_1 and lines were established for those candidates that continued to produce Egl progeny in

subsequent generations. In this way, we isolated five recessive mutations, four of which (*sy561*, *sy594*, *sy595*, and *sy596*) are linked to LGIV. The Egl phenotype of these alleles results from severe defects in vulval induction (average VPC induction in *sy561*, 1.4, $n = 30$; in s*y594*, 0.2, $n = 16$; in *sy595*, 0.2, $n = 12$; and in *sy596*, 0.1, $n = 19$). The mutant animals also exhibit embryonic and L1 stage lethality.

Complementation and mapping: *sli-3* was mapped to LGII on the basis of the following experiments. *sli-3* is not linked to *lin-3* since animals heterozygous for *sli-3 (sy341/+)* and *lin-3 (n378/+)* cosegregate only approximately one-quarter of the total progeny. Linkage tests with other markers were performed in the background of *lin-3(n378)* using vulval induction as an assay. Three-factor mapping using *rol-6(e187) unc-4(e120)* revealed that *sli-3* is likely to the right of *unc-4*. Specifically, all 8 Rol non-Unc recombinants picked up *sli-3(sy341)* whereas none of 7 Unc non-Rol picked up *sli-3(sy341)*. Futher mapping was done using *unc-4 let-25* and *unc-4 let-246* strains. *dpy-10 sy341; n378* hermaphrodites were mated with *unc-4 let-25/mnC1; n378* and *unc-4 let-246/mnC1; n378* males separately and viable Unc (recombinant) hermaphrodites were picked in the F_2 generation. All 19 Unc non-Let (let-25) recombinants picked up *sli-3(sy341)*, suggesting that *sli-3* is either to the right of *let-25* or very close to its left. Using *unc-4 let-246* we obtained 19 recombinants, 15 of which picked up *sli-3(sy341)*. Thus, *sli-3* is located between *unc-4* and *let-246*, closer to *let-246* than *unc-4*. We also used deficiencies to map the *sli-3* locus. The deficiencies *mnDf58* and *mnDf62* that uncover *let-25* and *let-246* fail to complement *sli-3(sy341)*. By contrast, deficiencies *mnDf61* and *mnDf29* that cover *let-25* and *let-246* complement *sli-3(sy341)*. Finally, deficiency *mnDf90* that uncovers *let-246* but not *let-25* also complements *sli-3(sy341)*.

The three *sli-4* alleles (*sy315*, *sy330*, and *sy339*) are considered allelic since they fail to complement each other when assayed for the suppression of *lin-3* Vul defect and confer similar phenotypes. The linkage was determined as follows. *let-312 lin-3(n378) unc-22/lin-3(n1059) sli-4 dpy-20* hermaphrodites were crossed to *lin-3(n378) dpy-20(e1282); him-5* males. F_1 Dpy progeny had wild-type vulval induction, and non-Dpy cross-progeny were all Vul, which indicates that *sli-4* is linked to *lin-3* and is dominant. For further mapping on LGIV, we crossed *n1059 sli-4 dpy-20/n378 dpy-20* hermaphrodites to *n378; him-5* males, and Dpy animals were picked out individually from *n1059 sli-4 dpy-20/n378*. When using *sy330*, 10 of 12 Dpy animals picked up *sy330*, and 8 of 10 picked up *sy339* when using *sy339*. In these experiments the presence of *sli-4* alleles was determined by the suppression of *n1059* lethality phenotype. The mapping results suggested that *sli-4* is between *lin-3* and *dpy-20* and close to *dpy-20*.

The *sli-4* revertants were linked to the *sli-4* chromosome (LGIV) on the basis of the following experiment. Hermaphrodites from the strain carrying *sy330* and one of the revertant alleles [*let-312(s1234) lin-3(n378) unc-22(s7)/lin-3(n1059) sli-4(sy330) dpy-20(e1282); revertant*] were crossed to *mec-3(e1338) him-8(e1489) dpy-20(e1282)/+* males. In the F_1 generation, Dpy males were crossed to *let-312(s1234) lin-3(n378)/DnT1; +/DnT1* hermaphrodites. Non-Unc F_2 worms were cloned and the progeny of those that did not segregate Dpy worms were examined for the Egl phenotype. The clones for all four *sli-4* revertants exhibited fully penetrant Egl phenotype, demonstrating their linkage to the *sli-4* chromosome. The deficiency mapping was carried out to further refine the genetic intervals of mutant loci. All four mutations complemented two deficiencies *sDf8* and *sDf62* and gave rise to viable progeny but not the deficiency *sDf63*. In this manner *sli-4* revertants were placed on LGIV very close to the *lin-3* locus. In the case of *sDf8* and *sDf62*, *mutant/Df* animals gave rise to viable progeny that exhibited wild-type vulval induction (data not shown). However, *mutant/sDf63* animals were embryonic/early L1 larval lethal, a phenotype that resembles *lin-3(n1059)* mutant animals. It should be pointed out that *sDf63* (but not *sDf8* and *sDf62*) uncovers the *lin-3* locus (Clark and Baillie 1992).

To determine linkage of *sli-5*, hermaphrodites of the genotype *n1059 dpy-20/let-312 n378 unc-22; sli-5* were crossed with *n378 dpy-20; him-5* males. Some animals of the resulting cross-progeny were non-Egl, suggesting that *sli-5* is not linked to *lin-3* and is semidominant for the suppression of the Vul phenotype of *lin-3*. We crossed *lin-3(n378); sli-5* males to hermaphrodites that carry Dpy or Unc markers, and, in F_2, Vul animals not displaying the marker phenotype were individually picked. In this manner, *sli-5* was assigned to linkage group X. From a three-factor mapping *sli-5* was mapped to the left of *lon-2*. Twenty-seven of 27 Unc non-Lon and 0 of 30 Lon non-Unc recombinant animals from *n378; lon-2 unc-97/sli-5* picked up *sli-5*.

On the basis of its map position, we tested whether the *sli-5(sy340)* mutation was a new allele of *sli-1* or *gap-1*, two previously identified negative regulators of vulval development that map to the left arm of the X chromosome (Yoon *et al.* 1995; Hajnal *et al.* 1997). *lin-3(n378); sli-5(sy340)* males were crossed into *lin-3(n378); unc-1 sli-1* or *dpy-5; lin-3(n378); gap-1 unc-2* hermaphrodites. *sli-5(sy340)* failed to complement both *sli-1* [2.78 cells induced ($n = 20$) for *sli-1/sli-5(sy340) vs.* 1.80 cells induced ($n = 20$) for *sli-1/+*] and *gap-1* [2.95 cells induced ($n = 20$) for *gap-1/sli-5(sy340) vs.* 1.93 cells induced for *gap-1/+* ($n = 22$)]. We have previously observed suppression of other *let-23* pathway mutations [*e.g.*, *let-23(sy1)*] by a *trans*-heterozygous combination of *sli-1* and *gap-1* alleles (data not shown). Thus, the complementation data are consistent with *sli-5(sy340)* being an allele of either locus.

Molecular analysis of *sli-4*: The genetic linkage mapping experiments revealed that all four *sli-4* revertants are tightly linked to *sli-4*, suggesting that they are either intragenic alleles or mutations in closely linked loci. Potential candidates in the *lin-3* genetic region include *let-60* (0.36 MU away from *lin-3*). Gain-of-function mutations in *let-60* (*e.g.*, *n1046dn*) are known to be epistatic to *lin-3* (Han *et al.* 1990). Since *let-60(dn)* alleles have been shown to alter the coding region of the gene (Beitel *et al.* 1990), we sequenced *let-60* RT–PCR products from each of the five *sli-4* revertants. None of the cDNA clones showed any mutation in *let-60* exons, suggesting that *let-60* is unaffected in mutant animals. To test the possibility that *sy330* revertants are allelic to *lin-3*, we sequenced the *lin-3* genomic region in *lin-3(n1059) sy330/lin-3(n378)* animals and detected a molecular change corresponding to *n378* (G61 to A) but none to *n1059* (G564 to A). Hence *sy330* represents a wild-type allele of *lin-3*. We also sequenced the *lin-3* genomic region in three of the revertant alleles (*sy561*, *sy594*, and *sy596*) that revealed mutations in the *lin-3* open reading frame. Two of these, *sy594* and *sy596*, have a premature stop codon (C595 to T and G80 to A, respectively) whereas *sy561* has a mutation identical to *n378* (G61 to A).

Analyses of the survival rate and fertility: Survival rate in different genotypes was calculated as described by Aroian and Sternberg (1991). Mutant hermaphrodites (for example, *unc-24 n1058/DnT1*) were mated with N2 males and F_1 non-Unc hermaphrodites (*unc-24 n1058/+* in this case) were individually picked and placed on plates. In F_2 the numbers of Unc and wild-type progeny were counted to determine the percentage of survival. For the N2 strain, survival was the ratio of the number of fertilized eggs and hatched progeny.

sli-3(sy341) animals have significantly low brood size compare to the wild type and are partially sterile. To determine whether these phenotypes are caused by *sy341* and not an unlinked mutation, we examined recombinant animals from a three-factor mapping cross with *unc-4 let-246* (~1.3 MU

interval). All 15 recombinants that suppressed the *lin-3(n378)* Vul defect (see above) showed reduced brood size. The tight association of the two phenotypes and their location within a small genetic interval (1.3 MU) suggest that the fertility defect is most likely caused by *sy341*. However, we cannot rule out the involvement of an unlinked very closely located mutation.

To understand the cellular basis of sterility in *sli-3(sy341)* animals, we examined the morphology of adult gonad and developing oocytes. In contrast to wild-type animals where oocytes in diakinesis appear almost square and are aligned in a row, *sy341* oocytes have abnormal morphology and do not align correctly. In addition, the number of oocytes in the gonad arms of 1- to 2-day-old *sy341* adults is significantly lower compared to the control (one gonad arm, 4.3 ± 1.9, $n = 20$; wild type, 7.3 ± 2.5, $n = 27$). While these defects do provide a partial explanation of the sterility in *sy341* animals, they do not rule out the possibility of an ovulation defect as an additional factor. To examine this possibility, we observed ovulation events in *sy341* animals under Nomarski optics but did not find any defect ($n = 4$ ovulations). Thus ovulation does not appear to be visibly compromised in *sli-3* mutants.

Molecular biology: *gap-1* and *sli-1* open reading frames (including introns) from *sli-5(sy340)* and wild-type N2 animals were amplified by PCR using primer pairs GL92/93 (*sli-1*: 5.6 kb) and GL94/95 (*gap-1*: 2.8 kb). *sli-1* DNA was sequenced using primers GL92, GL93, GL100, and GL101 and found to be wild type in *sy340* animals. Primers GL94, GL95, GL98, and GL99 were used to sequence the *gap-1* genomic DNA. We found one G-to-A mutation within a conserved 5′ splice site (GT) in intron 8 that prematurely introduces two in-frame stop codons (TGA and TAA). This was confirmed by sequencing both strands. Primer sequences are: GL92 (gccactggacttcacatcatatc acc), GL93 (cacaagtctactcccgctcactgttc), GL94 (atggttctatcttgca gagtcgtcgac), GL95 (cttctactcactttgttctccttctcg), GL98 (ggaaac cttcaacaagttgaccgaagc), GL99 (ctgacactacagttagacagcctttg), GL100 (gccaaattgcccaggtaattgaaac), and GL101 (gcaatgcaaagc atgcacattatctc).

RESULTS

Isolation of *lin-3* pathway regulators: To study mechanisms that regulate the LIN-3–LET-23-mediated vulval induction pathway in *C. elegans*, we carried out a novel genetic screen. The screen was designed to isolate suppressors of a severe, but nonnull *lin-3* phenotype. We used a strain (PS1031) that carries the hypomorphic *lin-3* allele *n378* in *trans* to the null allele *n1059* (see MATERIALS AND METHODS). While *n378* is a viable allele with significantly reduced vulval induction (Table 1), *n1059* is an embryonic/early L1 stage lethal (FERGUSON and HORVITZ 1985). We chose this genotype since none of the known viable alleles of *lin-3* (*e.g.*, *e1417*, *n378*, and *n1058*) are completely penetrant for the vulval induction defect (see Table 1). In *lin-3(n378)/lin-3(n1059)* heterozygous animals, vulval induction is severely reduced (average induction 0.03, $n = 29$) and all animals are egg-laying defective (Egl) (100%, $n = 244$). The severity of the vulval induction and egg-laying defects in this strain facilitated isolation of suppressors simply on the basis of the egg-laying defect. We expected to obtain two kinds of mutants from the screen: reduction-of-function (rf) alleles of negative regulators and gain-of-function (gf) alleles of positive regulators.

TABLE 1

Vulval induction in *lin-3* hypomorphs and suppressor alleles

Suppressor	*lin-3*	VPC induction[a]	n[b]
+	*e1417*	0.07 ± 0.18	20
+	*n378*	1.0 ± 1.1	43
+	*n1058*	1.4 ± 1.2	14
+	*n378/n1059*	0.03 ± 0.19	29
sy330/sy330	+	3.0 ± 0.0	15
sy340/sy340	+	3.0 ± 0.0	23
sy341/sy341	+	3.0 ± 0.0	50
sy330/+	*n378/n1059*	3.0 ± 0.0	30
sy340/sy340	*n378/n1059*	2.8 ± 0.5	12
sy340/+	*n378*	2.2 ± 0.9	14
sy341/sy341	*n378/n1059*	2.6 ± 0.4	8

The wild-type loci have been marked as "+."

[a] The average number of vulval precursors that acquire 1° and 2° cell fates and undergo cell divisions. The range is between 0 (no induction) and 6 (all VPCs induced). In wild-type animals 3.0 induction is observed. The values are shown as mean ± standard deviation.

[b] Number of L4 stage hermaphrodites examined.

F_2 progeny of EMS-mutagenized PS1031 animals were screened for suppressors that would revert the Egl phenotype. After screening nearly 30,000 haploid genomes, we isolated five mutations that suppress Egl and Vul defects of *lin-3(n378)/lin-3(n1059)* to almost wild-type levels (Table 1). Mutations were mapped to linkage groups using genetically marked strains and deficiencies (see MATERIALS AND METHODS). Complementation and mapping experiments revealed that three of the suppressors, *sy315*, *sy330*, and *sy339*, are allelic and define a single locus (*sli-4*) on LGIV. We chose *sy330* as a representative allele for further experiments. A combination of two- and three-factor mapping experiments helped localize *sli-4* on LGIV close to *dpy-20*. Of the remaining two suppressors, *sli-3(sy341)* maps on LGII either very close to the left of *let-25* (2.69 MU) or between *let-25* and *let-246* (2.98 MU) (Figure 1) and *sli-5(sy340)* maps on LGX to the left of *lon-2* (−6.70 MU) (see MATERIALS AND METHODS).

Characterization of *lin-3* suppressors: We examined the three classes of *lin-3* suppressor loci (*sli-3*, *sli-4*, and *sli-5*) by analyzing the ability of mutant alleles to suppress the *lin-3(n378)/lin-3(n1059)* Vul defect. *sli-3(sy341)* is a recessive loss-of-function allele by the following three criteria. First, *sy341/+* animals do not suppress the vulval induction defect in *lin-3(n378)* animals (average induction 1.3 ± 1.0, $n = 31$, $P = 0.2437$). Second, a duplication of the *sli-3* region *mnDp34* can suppress the *sy341* phenotype in the *lin-3(n378)* background (average induction 1.8 ± 1.2, $n = 25$ in *sy341/sy341, mnDp34* animals; $P = 0.1411$ when compared to *sy341/+*). Third, *sli-3(sy341)/Df* animals exhibit vulval induction comparable to that of *sli-3(sy341)* homozygotes in the *lin-3(n378)* genetic background (Table 2). The *sli-4* alleles (*sy315*, *sy330*, and

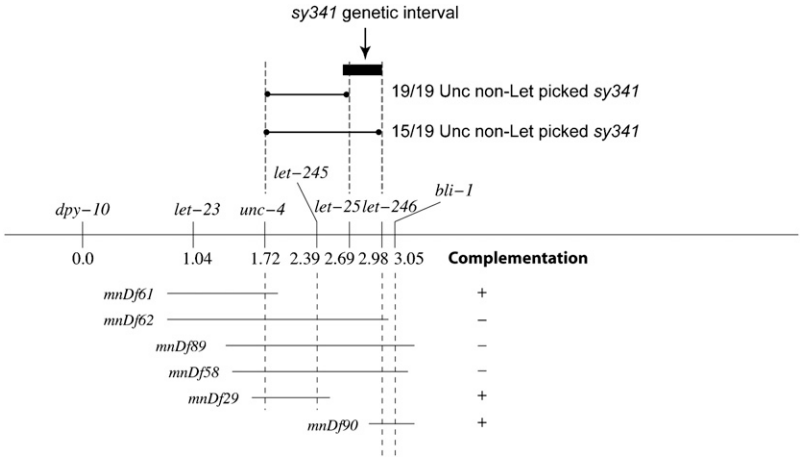

FIGURE 1.—A partial genetic map of *C. elegans* showing some of the markers and deficiencies used to define the interval of *sli-3*. The map positions of markers and extents of various deficiencies are indicated. The results of complementation tests between *sli-3(sy341)* and deficiencies have been marked with either + (*Df* complements) or − (*Df* does not complement). The three-factor mapping experiments place *sli-3* very close to the left of *let-25* or between *let-25* and *let-246*. In the case of the *unc-4 let-246* double, 19 of 19 Unc non-Let recombinants picked up *sli-3(sy341)*. Whereas, in the case of the *unc-4 let-25* double, 15 of 19 Unc non-Let recombinants picked up *sli-3(sy341)* (see MATERIALS AND METHODS for details).

sy339) are dominant suppressors of the *lin-3(n378)/lin-3(n1059)* Vul defect (Table 1). Finally, *sy340*, a single allele of *sli-5*, is semidominant as one copy of the mutation partially suppresses the vulval induction defect in *lin-3(n378)/lin-3(n1059)* animals (Table 1). All three classes of suppressor mutations are silent in an otherwise wild-type genetic background, suggesting that they are part of a redundantly acting regulatory network (Table 1).

Epistasis experiments revealed that *sli-4(sy330)* suppresses the Vul defect associated with *lin-3(rf)* but not *let-23(sy97)* (Table 3). Since the dominant nature of *sli-4* alleles (*sy315*, *sy330*, and *sy339*) did not allow further genetic studies, we sought to isolate recessive loss-of-function alleles by screening for *sy330* suppressors that exhibit a Vul phenotype. From a screen of 50,000 F_1's, we isolated four mutants—*sy561*, *sy594*, *sy595*, and *sy596*—that are phenotypically similar to *lin-3(n378)/lin-3(n1059)* and have almost zero vulval induction (see MATERIALS AND METHODS). Three of these (*sy594*, *sy595*, and *sy596*) also exhibit embryonic/early L1 larval stage lethality. The results of our three-factor and deficiency mapping as well as allele sequencing experiments (see MATERIALS AND METHODS) show that *sli-4* alleles correspond to the *lin-3* locus. Thus the dominant alleles of *sli-4* are intragenic revertants of *lin-3*, whereas the revertant alleles define new *lin-3* hypomorphs.

sli-5(sy340) strongly suppresses the *lin-3(rf)* Vul phenotype, suggesting that *sli-5* functions as a negative regulator of vulval induction. Consistent with this hypothesis, we found that *sy340* enhances the Muv phenotype of *let-23* gain-of-function allele *sa62* (Table 3). To further study *sli-5* in vulval development we carried out epistasis experiments with hypomorphs of the LET-23-mediated EGF signaling pathway. *sy340* suppresses the

TABLE 2

Deficiency mapping of *sli-3*

Genotype	*lin-3*	VPC induction	*n*
+	+	3.0 ± 0.0	>100
+	*n378*	1.0 ± 1.1	43
sy341/sy341	*n378*	3.0 ± 0.0	35
mnDf62/+	*n378*	1.3 ± 1.0	16
sy341/mnDf62	*n378*	2.4 ± 0.7 ($P = 0.0023$)	14
mnDf58/+	*n378*	1.3 ± 1.2	39
sy341/mnDf58	*n378*	2.5 ± 0.9 ($P < 0.0001$)	50
mnDf61/+	*n378*	1.4 ± 1.0	21
sy341/mnDf61	*n378*	1.0 ± 0.8 ($P = 0.1867$)	27
mnDf29/+	*n378*	1.7 ± 1.1	14
sy341/mnDf29	*n378*	1.2 ± 1.3 ($P = 0.3181$)	16
mnDf90/+	*n378*	1.2 ± 0.9	32
sy341/mnDf90	*n378*	1.0 ± 1.2 ($P = 0.5554$)	31

sli-3 is uncovered by two deficiencies, *mnDf62* and *mnDf58*. The other three deficiencies, *mnDf61*, *mnDf29*, and *mnDf90*, complement *sli-3*. VPC induction and "*n*" are defined in Table 1. The *P*-values are given in parentheses. The two groups of data are statistically significantly different if $P < 0.05$.

TABLE 3

Epistasis test of *lin-3* suppressor loci

Suppressors	EGF pathway genes	VPC induction	*n*
+	*let-23(sy97)*	0	21
sy330	*sy97*	0	27
+	*mpk-1(ku1)*	2.8 ± 0.7	22
sy330	*ku1*	2.3 ± 0.5 ($P = 0.09$)	22
sy340	*sy97*	2.9 ± 0.2	10
+	*let-23(sa62)*	3.1 ± 0.4[a]	14
sy340	*sa62*	4.3 ± 0.5[b] ($P < 0.000004$)	12
+	*let-60(n2034)*	0	11
sy340	*n2034*	2.3 ± 0.7	19
+	*lin-45(sy96)*	1.6 ± 1.1	29
sy340	*sy96*	0.4 ± 1.3	13

[a] Fourteen percent of animals exhibited a Muv phenotype. The genotype was *let-23(sa62) unc-4/mnC1; lon-2*.

[b] One hundred percent of animals exhibited a Muv phenotype. The genotype was *let-23(sa62) unc-4/mnC1; sy340 lon-2*. The *P*-values of average VPC induction are given where appropriate (the significance of data is defined in Table 2). The VPC induction and *n* are defined in Table 1.

Vul defect in *let-23(sy97)* and *let-60(n2034)* but not *lin-45(sy96)* animals (Table 3). Thus *sli-5* appears to function at the level of *let-60* during vulval induction. This property of *sli-5* resembles that of *gap-1*, which encodes a GTPase-activating protein for LET-60/Ras (Hajnal *et al.* 1997), and *sli-1*, an ortholog of c-Cbl, both of which map to the same region as *sli-5* (left of *lon-2* on LGX, see materials and methods). We carried out complementation tests with both *gap*-1 and *sli-1* and found that *sli-5* fails to complement both loci. We interpreted these data to suggest that *sli-5* was either *gap-1* or *sli-1* since we had previously found that a *trans*-heterozygous combination of a single *sli-1* and *gap-1* mutation can suppress hypomorphs in the *let-23* pathway (data not shown). Therefore, we sequenced the *gap-1* and *sli-1* genomic coding regions (including introns) in *sy340* animals. The sequence analysis identified a single G-to-A mutation in *gap-1*, while we failed to detect any mutations in *sli-1*. The G-to-A mutation is in the conserved splice donor site (GT) of intron 8 and is predicted to immediately add two consecutive STOP codons, which would prematurely truncate the protein after amino acid 497. This truncation would remove the last 12 amino acids of the PH domain, which might prevent GAP-1 from properly localizing with LET-60/Ras at the plasma membrane. On the basis of these results, we conclude that *sli-5* is a new allele of *gap-1*.

***sli-3* interacts with Ras-pathway genes in vulval cells:** The suppression of the *lin-3* Vul defect by *sy341* suggested that wild-type *sli-3* functions as a negative regulator of vulval development. Apart from the *lin-3* heteroallelic combination *n378/n1059*, we also tested the effect of *sli-3(sy341)* on two homozygous *lin-3* hypomorphs, *n378* and *n1058*. Vulval induction in these *lin-3* mutant animals is significantly reduced compared to that in the wild type (Table 1) and, in addition, *lin-3(n1058)* but not *lin-3(n378)* animals exhibit a fully penetrant sterile phenotype (Ferguson and Horvitz 1985; Clandinin *et al.* 1998). *sy341* suppresses the vulval defect in both *lin-3* alleles (Table 4) but not the sterile phenotype of *lin-3(n1058)* (see below and Table 8). To determine whether *sli-3*-mediated Vul suppression was limited to *lin-3*, we examined the ability of *sy341* to suppress vulval defects caused by mutations in other components of the LET-23 signaling pathway, including *let-23*, *let-60*, *lin-45*, and *mpk-1*. *sy341* suppresses the Vul defect associated with viable loss-of-function mutations in all of these genes. Thus, severe hypomorphic alleles of *let-23*, *let-60*, and *lin-45* are strongly suppressed by *sy341* (Table 4). The weak hypomorphs of *let-60* and *mpk-1* (*n2021* and *ku1*, respectively) are suppressed to wild-type levels (Table 4). These epistasis results suggest that *sli-3* negatively regulates the LET-23 signaling pathway during vulval development.

To examine the possibility of *sli-3* being a nuclear regulator of *let-23*-mediated signaling, we examined its interactions with known nuclear factors of the pathway.

TABLE 4

Epistasis test of *sli-3* with *let-23*-mediated EGF pathway genes

Genotype				
Vulval mutant	*sli-3*	% Egl	VPC induction	*n*
+	+	0 (>100)	3.0 ± 0.0	>100
lin-3(n1058)	+	NA	1.4 ± 1.2	14
n1058	*sy341*	NA	2.9 ± 0.3 ($P = 0.0002$)	14
lin-3(n378)	+	83 (72)	1.0 ± 1.1	43
n378	*sy341*[a]	3 (72)	3.0 ± 0.0	35
n378	*sy341*[b]	ND	2.7 ± 0.7	31
let-23(sy97)	+	100 (50)	0	20
sy97	*sy341*	46 (13)	1.7 ± 1.2	15
let-60(n1876)	+	100 (16)[c]	0	16
n1876	*sy341*	100 (6)[c]	2.2 ± 0.5	6
let-60(n2021)	+	21 (19)	2.6 ± 0.7	21
n2021	*sy341*	ND	3.0 ± 0.0 ($P = 0.0240$)	30
lin-45(sy96)	+	100 (60)	1.6 ± 1.1	29
sy96	*sy341*	100 (14)	2.9 ± 0.2 ($P = 0.0028$)	8
mpk-1(ku1)	+	ND	2.8 ± 0.7	22
ku1	*sy341*	ND	3.0 ± 0 ($P = 0.0471$)	37
lin-1(n1790gf)	+	69 (52)	2.9 ± 0.4[d]	36
n1790	*sy341*	48 (64)	3.3 ± 0.5[d] ($P = 0.0019$)	29
lin-1(n1761gf)	+	ND	2.7 ± 0.4	16
n1761	*sy341*	ND	3.0 ± 0.1 ($P = 0.0070$)	22
lin-25(e1446)	+	100 (136)	1.4 ± 0.4	20
e1446	*sy341*	100 (88)	1.6 ± 0.6 ($P = 0.2039$)	18
sur-2(ku9)	+	100 (45)	0.8 ± 0.6	25
ku9	*sy341*	100 (50)	1.4 ± 0.7 ($P = 0.0028$)	16

% Egl, percentage of egg-laying-defective animals; NA, not applicable; ND, not done.

For Egl phenotype the numbers in parentheses represent animals examined. VPC induction and *n* are defined in Table 1. The *P*-values of average VPC induction are given in parentheses (the significance of data is defined in Table 2).

[a] The genotype was *unc-4(e120) sli-3(sy341); lin-3(n378)*.

[b] The genotype was *dpy-10(e128) sli-3(sy341); lin-3(n378)*.

[c] The F_1 progeny of homozygous *let-60(n1876)* hermaphrodites die during the L1 stage (Beitel *et al.* 1990). *sy341* does not suppress larval lethality of *n1876* animals.

[d] *n1790* animals exhibit weak Muv phenotype (8%). The penetrance is higher (44%) in *sy341, n1790* double animals.

lin-1 encodes an ETS domain transcription factor that negatively regulates vulval induction (Beitel *et al.* 1990). We tested interactions of *sy341* with two gain-of-function alleles of *lin-1*, *n1761* and *n1790*, that cause an Egl phenotype due to reduced vulval induction and defective morphology (Beitel *et al.* 1990; Jacobs *et al.* 1998). The mutant *lin-1* alleles disrupt a conserved MAP kinase docking site in LIN-1, thereby making the altered protein unresponsive to MPK-1-mediated negative regulation

TABLE 5

Vulval cell lineages in *sli-3* and *sur-2* mutants

	VPC						
Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
sli-3(sy341)	S/SS	SS	LLTU	TTTT	UTLL	SS	10
sur-2(ku9)	SS	SS	SS	TTOO	UTS	SS	1
	SS	SS	SOU	OOOO	SS	SS	1
	SS	SS	SS	SS	SS	SS	3
	SS	SS	SS	UOO	SS	SS	2
	SS	SS	SOU	TTOO	SS	SS	1
	SS	S SS	SOU	TTOO	SS	S SS	1
ku9; sy341	S	SS	SUUO	TOOT	UOLL	S SS	1
	SS	SS	STTD	OOOT	SS	SS	1
	SS	SS	LLTU	TTLT	UUU	SS	1
	SS	S SS	LDDU	OTTO	UUU	SS	1
	SS	SS	LTUU	UOTO	SS	SS	1
	S	SS	UUU	OOTO	UOS	SS	1
	SS	SS	SS	SS	SS	SS	1
	SS	S SS	ssTU	ODOT	US	SS	1
	S	SS	LUUU	TTTO	ssss	SS	1

T, L, and O refer to transverse, longitudinal, and oblique axes of cell divisions of VPC granddaughters, respectively. D, cell division axis was not followed. U, undivided cell. S, syncytium fate after the first round of cell division of VPCs; s, syncytium fate after the second round of cell division of VPCs. *sur-2(ku9)* animals occasionally have extra VPCs. *n*, number of animals examined.

TABLE 6

Genetic interaction of *sli-3* with other negative regulators

Negative regulator	*sli-3*	% Muv	VPC induction	*n*
+	+	0	3.0 ± 0.0	>100
+	*sy341*	0	3.0 ± 0.0	50
sli-1(sy143)	+	0	3.0 ± 0.0	30
sy143	*sy341*	54	3.4 ± 0.4	22
ark-1(sy247)	+	6	3.0 ± 0.1	31
sy247	*sy341*	29	3.2 ± 0.4	51
unc-101(sy108)	+	3	3.0 ± 0.1	30
sy108	*sy341*	52	3.4 ± 0.4	21
gap-1(n1691)	+	0	3.0 ± 0.0	25
n1691	*sy341*	80	4.0 ± 0.7	15

The Muv phenotype and VPC induction were examined in L4 stage animals under a Nomarski microscope. Data for the *ark-1(sy247); sli-3(sy341)* double were pooled from two different genotypes, *dpy-10(e128) sli-3(sy341); ark-1(sy247) unc-31(e169)* (average induction = 3.2, Muv = 25%; $n = 24$) and *unc-4(e120) sli-3(sy341); dpy-20(e1282) ark-1(sy247)* (average induction = 3.2, Muv = 33%; $n = 27$). VPC induction and *n* are defined in Table 1.

(Jacobs *et al.* 1998). Analysis of the vulval phenotype in double-mutant animals revealed that *sli-3(sy341)* suppresses the induction defect in both *lin-1* alleles (Table 4). The Egl phenotype of *n1790* animals is also suppressed by *sy341* albeit only partially (Table 4). We also tested genetic interactions with mutations in two other nuclear regulators of the EGF signaling pathway, *lin-25* (novel) and *sur-2* (transcription mediator complex component). These gene products are essential for vulval induction and specify 1° and 2° cell fates (Singh and Han 1995; Tuck and Greenwald 1995; Stevens *et al.* 2002). Loss-of-function mutations in *lin-25* and *sur-2* result in the failure of VPCs to get induced, leading to a fully penetrant Egl phenotype. While *sy341* showed no obvious suppression of the VPC induction defect in *lin-25(e1446)* animals, the suppression of *sur-2(ku9)* phenotype is statistically significant (Table 4). The enhancement in vulval induction in *sur-2(ku9); sli-3(sy341)* double-mutant animals is almost entirely due to the higher frequency of the P5.p and P7.p precursors adopting the 2° cell fate (37%, $n = 32$ in double-mutant animals compared to 14%, $n = 50$ in *ku9* alone, where *n* stands for the total number of P5.p and P7.p VPCs scored). This is consistent with the analysis of the vulval cell lineages in mutant animals (Table 5). Taken together these results suggest that *sli-3* functions most likely either downstream or in parallel to *lin-1* and *sur-2* in vulval cells.

***sli-3* interacts with negative regulators of the inductive signaling pathway:** Two properties of *sli-3(sy341)* with regard to vulval induction strongly resemble mutations of previously identified negative regulators *sli-1*, *ark-1*, *unc-101*, and *gap-1* (Lee *et al.* 1994; Jongeward *et al.* 1995; Hajnal *et al.* 1997; Hopper *et al.* 2000). First, loss-of-function alleles of these genes have no effect on vulval induction and, second, mutations in each of these genes can suppress the Vul defect caused by mutations in the *let-23* pathway. Given the genetic similarity between *sli-3* and these negative regulators, we examined vulval phenotypes in double-mutant animals. *sli-3(sy341)* shows synergistic interactions with alleles of *sli-1*, *ark-1*, *unc-101*, and *gap-1* by giving rise to a multivulva (Muv) phenotype at significantly high frequencies (Table 6). The strongest interaction was observed with *gap-1* with 80% of the animals being Muv. The Muv phenotype was the result of ectopic induction in some or all of the P3.p, P4.p, and P8.p vulval precursors. Qualitative analysis of the Muv phenotypes, however, revealed some differences in the genetic interactions. We observed a high frequency of P8.p induction in *sli-3; gap-1* (64%, $n = 22$) and *unc-101; sli-3* (48%, $n = 21$) animals compared to *sli-3; ark-1* (11%, $n = 27$) and *sli-3; sli-1* (23%, $n = 22$) animals. *gap-1* was previously shown to preferentially induce P8.p in the background of *ark-1* mutation (Hopper *et al.* 2000). These results reveal functional differences between negative regulators in modulating the competence of different VPCs to respond to inductive signaling. This could help explain why there are so many different regulatory mechanisms.

In addition to the negative regulators discussed above, synthetic multivulva (SynMuv) genes are also known to inhibit vulval induction (Ferguson and Horvitz 1989). These genes are divided into three functionally redundant classes (A, B, and C) that negatively regulate

TABLE 7

VPC induction in *sli-3* and *lin-12* mutants

Growth temperature	*lin-12*	*sli-3*	% Muv	VPC induction
15°	*n137n460*	+	99 (167)	5.0 ± 0.7 (23)
20°	*n137n460*	+	99 (139)	4.8 ± 0.7 (20)
22°	*n137n460*	+	63 (155)	3.4 ± 0.5 (24)
	+	*sy341*	0 (20)	3.0 ± 0.0 (20)
	n137n460	*sy341*	50 (106)	3.9 ± 0.6 (22), $P = 0.0254$
25°	*n137n460*	+	3 (144)	3.0 ± 0.0 (30)
	+	*sy341*	0 (19)	3.0 ± 0.0 (19)
	n137n460	*sy341*	4 (57)	3.2 ± 0.4 (18), $P = 0.0206$

The Muv phenotype of the *lin-12* cold-sensitive allele, *n137n460*, varies with growth temperature. At 15° almost all animals exhibit Muv phenotype whereas at 25° rare animals do so. The phenotype was scored at plate level by looking for pseudovulvae (multiple ventral protrusions) in adult animals. Numbers in parentheses represent animals examined. The *P*-values are given where appropriate (the significance of data is defined in Table 2).

the fate of VPCs. We examined genetic interactions of *sli-3(sy341)* with class A and class B *lin-15* alleles, *n767* and *n744*, respectively. In each case >10 worms were examined but no Muv phenotype was observed. Thus *sli-3* appears to function in a genetic pathway separate from that mediated by *lin-15A* and *lin-15B* SynMuv genes during vulval induction.

***sli-3* does not interact with components of *lin-12/Notch* and *Wnt* signaling pathways:** In addition to LIN-3–LET-23-mediated inductive signaling, LIN-12-mediated lateral signaling also plays a crucial role in vulval induction (Greenwald 2005). Since our genetic experiments have demonstrated that *sli-3* acts as a negative regulator in both 1° and 2° lineage cells, we examined its interaction with *lin-12*. We used *lin-12(n137n460)*, a cold-sensitive allele of *lin-12*, which when grown at ≤20° gives rise to a fully penetrant Muv phenotype (Greenwald *et al.* 1983) (Table 7). The phenotype becomes progressively weaker at higher temperatures. Hence, while at 22° 63% ($n = 155$) of animals exhibit a Muv phenotype, at 25° *lin-12(n137n460)* animals are almost all wild type (3% Muv, see Table 7). We examined *sli-3(sy341); lin-12(n137n460)* double-mutant animals at 22° and 25° and found no enhancement in the Muv phenotype compared to the *lin-12(n137n460)* alone (Table 7). Thus *sli-3* is not likely to play a major role in *lin-12* signaling during establishment of the 2° lineage vulval fates.

We also examined genetic interactions of *sli-3* with two Wnt pathway components *bar-1*/β-*catenin* and *pry-1/axin* (Eisenmann *et al.* 1998; Eisenmann and Kim 2000; Gleason *et al.* 2002; Korswagen *et al.* 2002; Eisenmann 2005). Loss-of-function mutations in *bar-1* frequently cause cell fusion and induction defects in VPCs due to decreased LIN-39 activity (Eisenmann *et al.* 1998; Gleason *et al.* 2002). We examined vulval cells in *sli-3(sy341); bar-1(ga80)* double-mutant animals and observed a statistically significant increase in the number of induced VPCs (average induction 2.8 ± 0.4, $n = 39$ compared to 2.2 ± 0.7, $n = 41$ in *bar-1(ga80)* animals alone, $P < 0.0001$). This increase was accompanied by a suppression of the cell fusion defect in P5.p, P6.p, and P7.p (31, 2, and 14% suppression, respectively), suggesting that once the presumptive precursors are prevented from fusing, they are likely to get induced. Since the cell fusion defect in the *bar-1* mutant can be suppressed by elevated activity of the LET-60/Ras pathway (Eisenmann *et al.* 1998), we interpret this as an indirect effect of increased LET-60 pathway activity. In addition to reduced vulval induction, *bar-1(ga80)* animals also exhibit a P12 to P11 transformation defect (Eisenmann and Kim 2000). The penetrance of this phenotype is slightly reduced in a *sli-3(sy341)* genetic background [63%, $n = 39$ in double-mutant animals compared to 88%, $n = 41$ in *bar-1(ga80)* alone]. In contrast to *bar-1(lf)* animals that are Vul, hypomorphic alleles of *pry-1* (*e.g.*, *mu38*) exhibit a Muv phenotype (Gleason *et al.* 2002). We examined genetic interaction between *sli-3(sy341)* and *pry-1(mu38)* (at 22°) and found that Muv penetrance in *sy341, mu38* double-mutant animals (36%, $n = 39$) is not significantly different from that in *mu38* alone (28%, $n = 46$). Thus these results argue that *sli-3* is not a component of the Wnt signaling pathway during vulval induction.

***sli-3* does not participate in the IP_3R-mediated *let-23* fertility pathway:** Since *sli-3* is a regulator of *let-23*-mediated EGF signaling in vulval cells, we examined whether the same regulation might also occur in ovulation. In wild-type animals, ovulation depends upon the contraction of the gonadal sheath and requires IP_3-mediated calcium release (Clandinin *et al.* 1998; McCarter *et al.* 1999). The *n1058* allele of *lin-3* causes a fully penetrant sterile phenotype and partial defect in vulval induction (Tables 4 and 8). The sterility defect is due to the failure of the spermatheca to dilate correctly, thereby causing a defect in ovulation (Clandinin *et al.* 1998; Yin *et al.* 2004). We found that *sli-3(sy341); lin-3(n1058)* double-mutant animals are suppressed for the Vul defect but not the sterility (Tables 4 and 8). Hence, *sli-3* appears to regulate only a subset of *lin-3* functions. Alternatively, instead of being a negative regulator, *sli-3* may act positively in the fertility pathway. This would still be consistent with the observation that *sli-3(sy341)* animals have a reduced brood size and exhibit defective morphology of the oocytes (Table 8 and Figure 2; also see materials and methods). We thus examined genetic interaction of *sli-3* with gain-of-function mutations of *itr-1*, which encodes the IP_3 receptor and functions as an effector of LET-23 to control ovulation through dilation of the adult spermatheca (Clandinin *et al.* 1998). Mutations in *itr-1* suppress fertility defects in *lin-3(n1058)* animals, possibly by promoting ovulation

TABLE 8

Phenotypic analysis of *sli-3* mutants

Genotype	% viability[a]	%fertility[b]	Brood size[c]
+	96 (1226)	100 (>100)	252 ± 17 (7)
sli-3(sy341)	95 (179)	67 (70)	50 ± 22 (21)
lin-3(n378)	100 (100)	100 (72)	73 ± 20 (8)
sy341; n378	75 (138)	83 (72)	27 ± 13 (23)
lin-3(n1058)	ND	6 (16)	0.1 ± 0.5 (16)
sy341; n1058	ND	0 (14)	0 (14)
let-23(sy97)	21 (130)	95 (22)	ND
sy97 sy341	29 (115)	93 (14)	10 ± 5 (13)
let-60(n1876)	34 (80)	ND	0 (12)
sy341; n1876	47 (69)	ND	0 (6)
lin-45(sy96)	52 (150)	88 (8)	3 ± 2 (8)
sy341; sy96	62 (88)	85 (20)	1 ± 1 (6)

Numbers in parentheses represent animals examined for each phenotype. ND, not done.

[a] Survival was calculated as described in MATERIALS AND METHODS.

[b] Calculated as the percentage of hermaphrodites laying fertilized eggs.

[c] The number of progeny that survived to adulthood. Larvae that died during L1 and L2 stages were not counted.

(CLANDININ *et al.* 1998). The brood size of *sli-3(sy341); itr-1(sy290)* double-mutant animals (32 ± 14; $n = 12$) is not significantly different from that of *sy341* alone (50 ± 22; $n = 21$), suggesting that *sli-3* does not function in the IP_3-receptor-mediated *let-23* fertility pathway.

DISCUSSION

The *lin-3–let-23* signaling pathway plays a central role in the development of the vulva in *C. elegans* hermaphrodites (MOGHAL and STERNBERG 2003b; STERNBERG 2005). To identify additional genes that interact with this pathway and control vulval development, we carried out a genetic screen using a heteroallelic combination of two *lin-3* mutations, *n378* and *n1059*. In this article, we report the isolation of three suppressor loci and present a detailed analysis of one of these, *sli-3*. Our results demonstrate that *sli-3* is likely to function as a nuclear regulator of the LET-23/EGFR signaling pathway. The two other suppressors *sli-4* and *sli-5* are allelic to *lin-3* and *gap-1*, respectively.

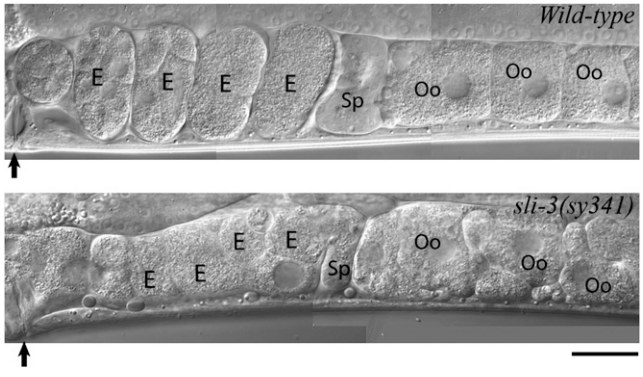

FIGURE 2.—*sli-3(sy341)* adult hermaphrodites exhibit defects in the morphology of oocytes and embryos. The arrows point to vulval opening. The spermatheca (Sp), embryos (E), and oocytes (Oo) are marked. In a wild-type animal (top), oocytes and embryos are aligned in a linear fashion. The *sy341* animal (bottom), on the other hand, exhibits no such arrangement. Bar, 30 μm.

Overview of the suppressor screen: Genetic screens in *C. elegans* have led to the successful identification of *let-23* pathway components that control vulval induction (STERNBERG 2005). Mutations in the core pathway genes were initially isolated on the basis of an Egl phenotype. These initial studies were followed by suppressor screens that identified additional pathway components as well as those that modulate pathway activity (*e.g.*, positive and negative regulators). Our screening strategy was similar to many others carried out in the past, except that, in contrast to the previous screens that involved alleles of *let-23*, *let-60*, *lin-10*, and *lin-15* (BEITEL *et al.* 1990; HAN *et al.* 1990, 1993; HAJNAL *et al.* 1997; MOGHAL and STERNBERG 2003b), we used alleles of *lin-3*.

We recovered a new locus *sli-3*, identified by a suppressor of the *lin-3* Vul phenotype, that functions as a negative regulator of vulval induction. Several properties of *sli-3* closely resemble those of previously identified negative regulators *sli-1*, *ark-1*, *unc-101*, and *gap-1* (MOGHAL and STERNBERG 2003b). Mutations in any of these genes alone do not exhibit a visible vulval defect but double-mutant combinations show synergistic interactions resulting in a Muv phenotype (STERNBERG 2005; SUNDARAM 2005). Except for *gap-1*, our genetic screen did not recover alleles of known negative regulators, perhaps due to the smaller number of genomes screened. Compared to the previous genetic screens that were carried out at a larger scale (>100,000 haploid genome sets in the case of *sli-1* and *gap-1* in standard F_2 generation screens and ~25,000 in the case of the *unc-101* synthetic enhancement screen), we screened fewer animals (~30,000; see MATERIALS AND METHODS). The fact that we recovered only one allele of *sli-3* also supports this possibility. However, we cannot rule out an intrinsic bias in our screen toward recovering alleles of certain genes. Our genetic epistasis experiments have demonstrated that compared to other negative regulators that function either at the level of the LET-23 receptor (SLI-1, ARK-1, and UNC-101) or at that of LET-60/Ras (GAP-1) (LEE *et al.* 1994; JONGEWARD *et al.* 1995; HOPPER *et al.* 2000), SLI-3 is the most downstream acting negative regulator of vulval induction identified thus far in *C. elegans*.

In addition to the alleles of *sli-3* and *sli-5*, two extragenic suppressor loci, we also recovered *sli-4* mutants that are allelic to *lin-3*. Our initial mapping experiments had suggested that *sli-4* defines a new locus close to *lin-3*. This was due to the presence of an unidentified lethal

mutation that, similar to *lin-3(n1059)*, causes embryonic/early larval stage lethality. Since we relied upon the *lin-3(n1059)* larval lethality phenotype to map *sli-4* (see MATERIALS AND METHODS), the three-factor mapping experiments led us to conclude that *sli-4* is genetically separate from *lin-3*. We do not know whether the lethal mutation arose spontaneously or due to the EMS treatment. The preliminary mapping experiments indicated tight linkage to *lin-3* (~1.8 MU to the left of *lin-3*).

SLI-3 is a tissue-specific regulator of the LET-23/EGFR signaling pathway: The *let-23* EGF receptor system is required for the development of multiple tissues in *C. elegans* (MOGHAL and STERNBERG 2003b). Among at least five different roles of the pathway components identified so far, four (viability, vulval induction, P12 cell, and male tail spicule developments) are regulated by *let-60/ras*, whereas the fertility process utilizes IP_3-mediated Ca^{2+} signaling. The presence of common *let-23* signaling pathway components in multiple developmental processes suggests that functional specificity is likely to be mediated by tissue-specific downstream effectors/regulators. If so, carefully designed genetic screens should be able to identify such tissue-specific components. Our analysis of *sli-3* function has revealed that *sli-3* specifically functions in the *let-23*-mediated vulval induction pathway. Thus, the *sli-3* mutation does not suppress nonvulval defects associated with *let-23* pathway genes. The reduced viability and fertility defects in hypomorphic alleles of *let-23*, *let-60*, and *lin-45* are significantly not altered in a *sy341* background (Table 8). Furthermore, the P12 fate specification defect in *let-23(sy97)* animals is also not suppressed (data not shown).

The genetic analysis of *let-23*-mediated signaling in hermaphrodite fertility has revealed that some of the pathway components function in two distinct processes: germline development (cell cycle progression) and ovulation (spermathecal contraction). While *let-60*, *mpk-1*, and *mek-1* play crucial roles in germline development (CHURCH *et al.* 1995), genes such as *lin-3*, *let-23*, *itr-1*, and *lfe-2* are involved in the ovulation process (CLANDININ *et al.* 1998). Mutations in the former set of genes cause sterility due to the arrest of germ cell nuclei in the pachytene stage. On the other hand, mutations in the latter set of genes disrupt dilation of the spermatheca, leading to an ovulation defect. To determine whether *sli-3* participates in any one or both of these processes, we tested its requirement in germline development and in ovulation by examining phenotypes of the mutant animals as well as by genetic interaction studies. Three experiments suggest a distinct role of *sli-3* in hermaphrodite fertility. First, *sy341* animals show neither an ovulation defect nor a diakinesis-stage arrest of germ cells (see MATERIALS AND METHODS). Second, *sli-3* shows no discernible genetic interaction with *itr-1*, suggesting that *sli-3* is not a component of IP_3 signaling during oocyte maturation and ovulation. Third, *sli-3(sy341)* does not enhance fertility defects of EGF pathway mutants (Table 8). Since *sy341* hermaphrodites have morphologically defective oocytes and the sterility defect in mutant animals can be partially rescued by mating with wild-type males (data not shown), *sli-3* is likely to play a role in gametogenesis.

***sli-3* is a *let-23/EGFR* pathway-specific effector in vulval cells:** In *C. elegans*, vulval development is controlled by three evolutionarily conserved signaling pathways, namely LET-23/EGFR, LIN-12/Notch, and Wnt. Given that these pathways are also required for the development and patterning of other tissues, their specific responses are likely to be mediated by pathway components that possess cell- and/or tissue-specific activities. Our experiments have revealed that *sli-3* negatively regulates the LET-23/EGFR signaling pathway in the vulva. In the absence of any other mutation, *sli-3(sy341)* animals exhibit wild-type vulval induction, suggesting that *sli-3* is dispensable for normal development. The gene dosage studies reveal that the suppression of the *lin-3(n378)* VPC induction defect by *sy341* can be ranked as follows (starting from the highest): *sy341/sy341* > *sy341/Df* > *sy341/sy341/+(mnDp34)* ≈ *sy341/+*. This is most consistent with *sy341* being a hypomorph.

Our epistasis experiments have revealed that the *sli-3* mutation suppresses Vul defects caused by viable hypomorphic alleles of *let-23*, *let-60*, *lin-45*, and *mpk-1*. Similar epistasis experiments with nuclear targets of the *let-23* signaling pathway have revealed that the *sli-3* mutation can suppress the Vul defect caused by gain-of-function alleles of *lin-1* and a severe hypomorphic allele of *sur-2*, genes that encode ETS domain protein and a component of the transcription mediator complex, respectively (BEITEL *et al.* 1995; SINGH and HAN 1995; STEVENS *et al.* 2002). These results indicate that *sli-3* most likely functions in the nucleus either downstream or in parallel to *lin-1* and *sur-2* to specify 1° and 2° fates to VPCs. Furthermore, instead of being a major target of the *let-23* pathway, *sli-3* defines a regulatory branch of the signaling. The lineage analysis of *sur-2* and *sli-3* double-mutant animals has revealed a higher frequency of 2° cell fate specification (see Table 5). Since SUR-2 facilitates the crosstalk between the inductive and lateral signaling pathways to specify 2° fates to P5.p and P7.p (SHAYE and GREENWALD 2002), one possibility could be that SLI-3 mediates SUR-2 function during this specification process. In addition, SLI-3 may be regulated by LIN-1 to confer 1° fates on the VPCs. The molecular identity of *sli-3* will help test these hypotheses and its precise mechanism of function in vulval cells.

To determine whether *sli-3* is a pathway-specific effector, we examined its interactions with genes that encode components of LIN-12/Notch and Wnt signaling pathways. We found that *sli-3* does not show strong genetic interactions with the *lin-12*, *bar-1*, and *pry-1* alleles tested, suggesting that it may not function as a

common regulator of multiple signaling pathways. The involvement of *sli-3* in mediating *let-23* pathway function in the vulva demonstrates that similar mechanisms are likely to exist in other tissues to provide specificity to *let-60*-mediated EGF signaling in *C. elegans*.

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LITERATURE CITED

Aroian, R. V., and P. W. Sternberg, 1991 Multiple functions of let-23, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. Genetics **128:** 251–267.

Beitel, G. J., S. G. Clark and H. R. Horvitz, 1990 Caenorhabditis elegans ras gene let-60 acts as a switch in the pathway of vulval induction. Nature **348:** 503–509.

Beitel, G. J., S. Tuck, I. Greenwald and H. R. Horvitz, 1995 The Caenorhabditis elegans gene lin-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes Dev. **9:** 3149–3162.

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

Christensen, S., V. Kodoyianni, M. Bosenberg, L. Friedman and J. Kimble, 1996 lag-1, a gene required for lin-12 and glp-1 signaling in Caenorhabditis elegans, is homologous to human CBF1 and Drosophila Su(H). Development **122:** 1373–1383.

Church, D. L., K. L. Guan and E. J. Lambie, 1995 Three genes of the MAP kinase cascade, mek-2, mpk-1/sur-1 and let-60 ras, are required for meiotic cell cycle progression in Caenorhabditis elegans. Development **121:** 2525–2535.

Clandinin, T. R., J. A. DeModena and P. W. Sternberg, 1998 Inositol trisphosphate mediates a RAS-independent response to LET-23 receptor tyrosine kinase activation in C. elegans. Cell **92:** 523–533.

Clark, D. V., and D. L. Baillie, 1992 Genetic analysis and complementation by germ-line transformation of lethal mutations in the unc-22 IV region of Caenorhabditis elegans. Mol. Gen. Genet. **232:** 97–105.

Clark, D. V., T. M. Rogalski, L. M. Donati and D. L. Baillie, 1988 The unc-22(IV) region of *Caenorhabditis elegans*: genetic analysis of lethal mutations. Genetics **119:** 345–353.

Eisenmann, D. M., 2005 Wnt signaling (January 1, 2006), pp. 1–17 in *WormBook*, edited by I. Greenwald (http://www.wormbook.org).

Eisenmann, D. M., and S. K. Kim, 2000 Protruding vulva mutants identify novel loci and Wnt signaling factors that function during *Caenorhabditis elegans* vulva development. Genetics **156:** 1097–1116.

Eisenmann, D. M., J. N. Maloof, J. S. Simske, C. Kenyon and S. K. Kim, 1998 The β-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during Caenorhabditis elegans vulval development. Development **125:** 3667–3680.

Ferguson, E. L., and H. R. Horvitz, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. Genetics **110:** 17–72.

Ferguson, E. L., and H. R. Horvitz, 1989 The multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. Genetics **123:** 109–121.

Garriga, G., C. Desai and H. R. Horvitz, 1993 Cell interactions control the direction of outgrowth, branching and fasciculation of the HSN axons of Caenorhabditis elegans. Development **117:** 1071–1087.

Gleason, J. E., H. C. Korswagen and D. M. Eisenmann, 2002 Activation of Wnt signaling bypasses the requirement for RTK/Ras signaling during C. elegans vulval induction. Genes Dev. **16:** 1281–1290.

Greenwald, I., 2005 LIN-12/Notch signaling in C. elegans (August 4, 2005), pp. 1–15 in *WormBook*, edited by L. R. Girard (www.wormbook.org).

Greenwald, I. S., P. W. Sternberg and H. R. Horvitz, 1983 The lin-12 locus specifies cell fates in Caenorhabditis elegans. Cell **34:** 435–444.

Hajnal, A., C. W. Whitfield and S. K. Kim, 1997 Inhibition of Caenorhabditis elegans vulval induction by gap-1 and by let-23 receptor tyrosine kinase. Genes Dev. **11:** 2715–2728.

Han, M., R. V. Aroian and P. W. Sternberg, 1990 The let-60 locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*. Genetics **126:** 899–913.

Han, M., A. Golden, Y. Han and P. W. Sternberg, 1993 C. elegans lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation. Nature **363:** 133–140.

Herman, R. K., 1978 Crossover suppressors and balanced recessive lethals in *Caenorhabditis elegans*. Genetics **88:** 49–65.

Herman, R. K., J. E. Madl and C. K. Kari, 1979 Duplications in *Caenorhabditis elegans*. Genetics **92:** 419–435.

Hodgkin, J., H. R. Horvitz and S. Brenner, 1979 Nondisjunction mutants of the nematode *Caenorhabditis elegans*. Genetics **91:** 67–94.

Hopper, N. A., J. Lee and P. W. Sternberg, 2000 ARK-1 inhibits EGFR signaling in C. elegans. Mol. Cell **6:** 65–75.

Horvitz, H. R., S. Brenner, J. Hodgkin and R. K. Herman, 1979 A uniform genetic nomenclature for the nematode Caenorhabditis elegans. Mol. Gen. Genet. **175:** 129–133.

Jacobs, D., G. J. Beitel, S. G. Clark, H. R. Horvitz and K. Kornfeld, 1998 Gain-of-function mutations in the Caenorhabditis elegans lin-1 ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. Genetics **149:** 1809–1822.

Jongeward, G. D., T. R. Clandinin and P. W. Sternberg, 1995 sli-1, a negative regulator of let-23-mediated signaling in *C. elegans*. Genetics **139:** 1553–1566.

Korswagen, H. C., D. Y. Coudreuse, M. C. Betist, S. van de Water, D. Zivkovic *et al.*, 2002 The Axin-like protein PRY-1 is a negative regulator of a canonical Wnt pathway in C. elegans. Genes Dev. **16:** 1291–1302.

Lee, J., G. D. Jongeward and P. W. Sternberg, 1994 unc-101, a gene required for many aspects of Caenorhabditis elegans development and behavior, encodes a clathrin-associated protein. Genes Dev. **8:** 60–73.

Maloof, J. N., J. Whangbo, J. M. Harris, G. D. Jongeward and C. Kenyon, 1999 A Wnt signaling pathway controls hox gene expression and neuroblast migration in C. elegans. Development **126:** 37–49.

McCarter, J., B. Bartlett, T. Dang and T. Schedl, 1999 On the control of oocyte meiotic maturation and ovulation in Caenorhabditis elegans. Dev. Biol. **205:** 111–128.

Moerman, D. G., and D. L. Baillie, 1979 Genetic organization in *Caenorhabditis elegans*: fine-structure analysis of the *unc-22* gene. Genetics **91:** 95–103.

Moghal, N., and P. W. Sternberg, 2003a A component of the transcriptional mediator complex inhibits RAS-dependent vulval fate specification in C. elegans. Development **130:** 57–69.

Moghal, N., and P. W. Sternberg, 2003b The epidermal growth factor system in Caenorhabditis elegans. Exp. Cell Res. **284:** 150–159.

Shaye, D. D., and I. Greenwald, 2002 Endocytosis-mediated downregulation of LIN-12/Notch upon Ras activation in Caenorhabditis elegans. Nature **420:** 686–690.

Sigurdson, D. C., G. J. Spanier and R. K. Herman, 1984 *Caenorhabditis elegans* deficiency mapping. Genetics **108:** 331–345.

Singh, N., and M. Han, 1995 sur-2, a novel gene, functions late in the let-60 ras-mediated signaling pathway during Caenorhabditis elegans vulval induction. Genes Dev. **9:** 2251–2265.

Sternberg, P. W., 2005 Vulval development (June 25, 2005), pp. 1–28 in *Wormbook*, edited by B. J. Meyer (http://www.wormbook.org).

Stevens, J. L., G. T. Cantin, G. Wang, A. Shevchenko, A. Shevchenko *et al.*, 2002 Transcription control by E1A and MAP kinase pathway via Sur2 mediator subunit. Science **296:** 755–758.

Sulston, J. E., and H. R. Horvitz, 1977 Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. **56:** 110–156.

Sundaram, M., 2005 RTKRas/MAP kinase signaling, pp. 1–19 in *Wormbook* (http://www.wormbook.org).

Tuck, S., and I. Greenwald, 1995 lin-25, a gene required for vulval induction in Caenorhabditis elegans. Genes Dev. **9:** 341–357.

Yin, X., N. J. Gower, H. A. Baylis and K. Strange, 2004 Inositol 1,4,5-trisphosphate signaling regulates rhythmic contractile activity of myoepithelial sheath cells in Caenorhabditis elegans. Mol. Biol. Cell **15:** 3938–3949.

Yoon, C. H., J. Lee, G. D. Jongeward and P. W. Sternberg, 1995 Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c–cbl. Science **269:** 1102–1105.

Zengel, J. M., and H. F. Epstein, 1980 Identification of genetic elements associated with muscle structure in the nematode Caenorhabditis elegans. Cell Motil. **1:** 73–97.

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