A *lin-45 raf* Enhancer Screen Identifies *eor-1*, *eor-2* and Unusual Alleles of Ras Pathway Genes in *Caenorhabditis elegans*

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Manuscript received December 17, 2001
Accepted for publication February 11, 2002

ABSTRACT

In *Caenorhabditis elegans*, the Ras/Raf/MEK/ERK signal transduction pathway controls multiple processes including excretory system development, P12 fate specification, and vulval cell fate specification. To identify positive regulators of Ras signaling, we conducted a genetic screen for mutations that enhance the excretory system and egg-laying defects of hypomorphic *lin-45 raf* mutants. This screen identified unusual alleles of several known Ras pathway genes, including a mutation removing the second SH3 domain of the *sem-5*/Grb2 adaptor, a temperature-sensitive mutation in the helical hairpin of *let-341*/Sos, a gain-of-function mutation affecting a potential phosphorylation site of the *lin-1* Ets domain transcription factor, a dominant-negative allele of *ksr-1*, and hypomorphic alleles of *sur-6*/PP2A-B, *sur-2*/Mediator, and *lin-25*. In addition, this screen identified multiple alleles of two newly identified genes, *eor-1* and *eor-2*, that play a relatively weak role in vulval fate specification but positively regulate Ras signaling during excretory system development and P12 fate specification. The spectrum of identified mutations argues strongly for the specificity of the enhancer screen and for a close involvement of *eor-1* and *eor-2* in Ras signaling.

RECEPTOR tyrosine kinase (RTK)-Ras-extracellular signal regulated kinase (ERK) signaling pathways control many different aspects of animal development. The basic features of such signaling pathways have now been elucidated through a combination of biochemical studies in mammalian cells and genetic studies in model organisms such as Drosophila and *Caenorhabditis elegans* (Wassarman *et al.* 1995; Campbell *et al.* 1998; Sternberg and Han 1998; Vojtek and Der 1998). Growth factor binding stimulates dimerization and subsequent autophosphorylation of RTKs, creating docking sites for adaptor proteins such as Grb2 (Schlessinger 2000). Grb2 binds to RTK phosphotyrosine sites via its SH2 domain and to the guanine nucleotide exchange factor (GEF) Sos via its SH3 domains. This interaction localizes Sos to the plasma membrane and allows it to catalyze the exchange of GDP for GTP on Ras (Downward 1996). Ras-GTP then binds to the kinase Raf, and other poorly understood events at the plasma membrane stimulate Raf kinase activity (Morrison and Cutler 1997). Once activated, Raf phosphorylates and activates MEK, which then phosphorylates and activates ERK. ERK can then translocate into the nucleus where it phosphorylates multiple substrates, including Ets domain transcription factors (Yordy and Muise-Helmericks 2000), which then cooperate with or antagonize other factors to elicit cell-type-specific responses (Tan and Kim 1999; Simon 2000). Since misregulated Ras pathway signaling contributes to many human pathologies, including cancer, it is of great interest to understand the different ways in which this pathway is normally regulated and might be therapeutically controlled. To identify positive regulators of Ras signaling, we conducted a genetic screen for enhancers of *lin-45 raf* mutant defects in *C. elegans*.

In *C. elegans*, Ras signaling is conveniently not required for mitotic cell division during larval development (Yochem *et al.* 1997), but it is required for multiple developmental events, including excretory duct cell fate specification (and hence viability; Yochem *et al.* 1997), germline meiotic progression (and hence fertility; Church *et al.* 1995), the P12 ectodermal blast cell fate (Jiang and Sternberg 1998), proper sex myoblast migration (Sundaram *et al.* 1996), and certain gonadal and vulval cell fates (and hence egg-laying ability; Han *et al.* 1990; Chang *et al.* 1999; Wang and Sternberg 2000). The Ras pathway has been best studied for its role in vulval fate specification, where it determines in part which of six initially equipotent vulval precursor cells (VPCs) will adopt vulval fates (Sternberg and Han 1998). In wild-type animals, three VPCs adopt vulval fates. Activating mutations in Ras pathway genes cause a Multivulva (Muv) phenotype in which more than three VPCs adopt vulval fates, while loss-of-function mutations in Ras pathway genes cause a Vulvaless (Vul) phenotype in which fewer than three VPCs adopt vulval fates.

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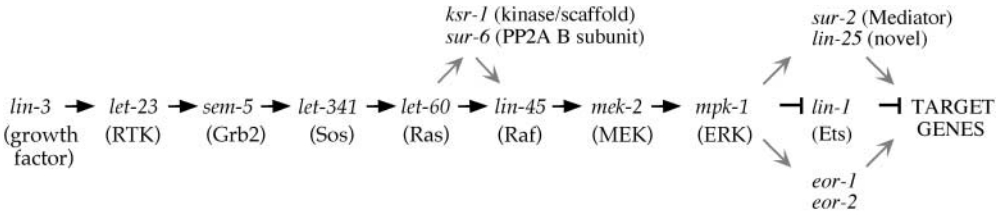

FIGURE 1.—The core Ras pathway in *C. elegans* (STERNBERG and HAN 1998) and positive regulators identified in the *lin-45 raf* enhancer screen. *C. elegans* genes are shown in italics. Molecular identities or mammalian homologs are indicated in parentheses. Some tissue-specific targets of the pathway, such as *lin-31* (MILLER *et al.* 1993) and *lin-39* (MALOOF and KENYON 1998), are not shown. *eor-1* and *eor-2* are placed downstream or in parallel to *mpk-1* on the basis of epistasis analysis and molecular identities (R. M. HOWARD and M. V. SUNDARAM, unpublished results).

Screens for Muv and Vul mutants (or for suppressors of such mutants) have elucidated a core Ras pathway consisting of *lin-3* (epidermal growth factor-like growth factor), *let-23* (RTK), *sem-5* (Grb2), *let-341* (Sos), *let-60* (Ras), *lin-45* (Raf), *mek-2* (MEK), *mpk-1* (ERK), and *lin-1* (Ets domain; Figure 1). Such screens have also identified genes that regulate signal transmission through the Ras pathway, including *ksr-1* (KORNFELD *et al.* 1995; SUNDARAM and HAN 1995), and several likely transcriptional regulators that appear to act in parallel to *lin-1*, including *sur-2* (SINGH and HAN 1995) and *lin-25* (TUCK and GREENWALD 1995; Figure 1).

Since most screens for Ras pathway regulators have focused on vulval phenotypes, genes that primarily regulate Ras signaling in other tissues likely have been missed. By screening for enhancers of the egg-laying defective (Egl) and lethal defects of hypomorphic *lin-45 raf* mutants, we identified multiple alleles of *eor-1* and *eor-2*, two genes that positively regulate Ras signaling during excretory system development and P12 fate specification and play a relatively minor role during vulval development. These genes were identified independently in other genetic screens (M. HERMAN and M. HENGARTNER, personal communication) and hence named *eor* (**e***gl-1* suppressor, Di**O**-uptake defective, **R**af enhancer). In addition, we identified interesting alleles of several known Ras pathway genes, including *sem-5*, *let-341*, and *lin-1*. The genetic behavior and molecular lesions of these alleles provide insight into the normal regulation of Ras pathway components.

MATERIALS AND METHODS

General methods and alleles: General methods for the handling, culturing, and ethyl methanesulfonate (EMS) mutagenesis of nematodes were as previously described (BRENNER 1974). Experiments were performed at 20° unless otherwise noted. *C. elegans var* Bristol strain N2 is the wild-type parent for all strains used in this work. Specific genes and alleles are listed below (see RIDDLE *et al.* 1997 and references therein unless otherwise noted).

LGI: *dpy-5(e61)*, *sur-6(cs24)* (SIEBURTH *et al.* 1999), *unc-13(e51)*, *unc-54(e190)*, *unc-101(m1)*.

LGIV: *dpy-13(e184)*, *dpy-20(e1282)*, *let-60(n1046gf)*, *let-277(m262)*, *let-279(m261)*, *let-280(m259)*, *let-281 (m247)*, *let-282(m258)*, *let-284(m267)*, *lin-45(ku51)* (Y. HAN and M. HAN, personal communication), *lin-45(ku112)* (SUNDARAM and HAN 1995), *mDf8* (ROGALSKI and RIDDLE 1988), *unc-5(e53)*, *unc-17(e113)*.

LGV: *dpy-11(e224)*, *eT1(III;V)* (ROSENBLUTH and BAILLIE 1981), *let-341(s1031)*, *lin-25(e1446)*, *unc-46 (e177)*, *unc-76(e936)*.

LGX: *dpy-6(e14)*, *gap-1(ga133)* (HAJNAL *et al.* 1997), *ksr-1(n2526)*, *let-4(mn105)*, *lin-15(n765)*, *lon-2 (e678)*, *sem-5(n1779)*, *syc-2 (gm132)* (FORRESTER *et al.* 1998), *unc-3(e151)*, *mnDf7* (MENEELY and HERMAN 1979).

Isolation and preliminary characterization of enhancer mutations: MH575 [*lin-45(ku51) dpy-20*] or MH620 [*lin-45(ku112) dpy-20*] hermaphrodites were mutagenized with 50 mM EMS and allowed to self-fertilize; their F_1 progeny were then picked to individual plates and allowed to self-fertilize. From plates with multiple F_2 Egl animals, individual Egl animals were picked to screen for the presence of rod-like arrested larvae inside the body cavity (bag of rods phenotype) and to establish candidate *lin-45 dpy-20; m/m* homozygous lines (where *m* is the new mutation). In cases where the penetrance of the Egl and lethal phenotypes was very high, wild-type siblings were also picked to establish heterozygous lines. Mutations isolated in the *ku51* background include *cs1*, *cs7*, *cs8*, *cs13*, and *cs14* (*cs14* was subsequently lost). Mutations isolated in the *ku112* background include *cs15*, *cs24*, *cs26*, *cs28*, *cs30*, *cs31*, *cs40*, *cs41*, *cs42*, *cs43*, *cs44*, *cs47*, *cs48*, *cs50*, *cs51*, and *cs52*.

Candidate strains were outcrossed to N2 males, and the broods of *lin-45 dpy-20/++; m/+* animals were scored to determine whether *m* was an enhancer of *lin-45 raf*. If comparable proportions of Dpy and non-Dpy animals were Egl, then *m* was inferred to cause an Egl phenotype on its own, and non-Dpy Egl animals were picked to establish putative *m/m* lines for further outcrossing and analysis. Alleles in this category include *sur-2(cs8, cs43, cs48)*, *lin-25(cs13)*, *sem-5(cs15)*, and five others that resemble *sur-2* or *lin-25* alleles but have not been further characterized. If Dpy animals were more frequently Egl than non-Dpy animals, then *m* was inferred to be an enhancer mutation (or else linked to *dpy-20*), and candidate *lin-45 dpy-20/++; m/m* animals were identified and used to establish putative *m/m* lines for further outcrossing and analysis. Each mutation was then crossed back into the *lin-45* mutant background to verify its enhancer properties.

Mapping and complementation tests: Genetic mapping and complementation tests were performed using standard methods. With the exception of *lin-1(cs50)*, all mutations described here are recessive enhancers.

sem-5(cs15) X: cs15 was initially mapped to the X chromosome on the basis of the observation that male cross-progeny of *cs15* mothers were incapable of mating. Of 42 *cs15* homozygotes from *cs15/lon-2 dpy-6* mothers, 10 segregated *lon-2* and one segregated *dpy-6*. *cs15* failed to complement *sem-5(n1779)* for the Egl phenotype.

let-341(cs41) V: Two-factor mapping experiments were performed in the *lin-45(ku112)* background at 20°. Of 10 *cs41* homozygotes from *cs41/dpy-11* mothers, 3 segregated *dpy-11*.

At 25°, *cs41* failed to complement *let-341(s1031)* for the Egl and lethal phenotypes.

lin-1(cs50) IV: In the initial outcross of *cs50 lin-45(ku112) dpy-20*, >25% (35/95) of the Dpy animals but almost no non-Dpy animals (1/161) were Egl, suggesting that *cs50* is on chromosome IV. Of 73 *cs50 lin-45(ku112)* homozygotes from *cs50 lin-45(ku112)/unc-17 dpy-13* mothers, none segregated *unc-17* and/or *dpy-13*. *cs50* was determined to be an allele of *lin-1* by DNA sequencing.

ksr-1(cs1) X: cs1 was mapped to the X chromosome on the basis of the observation that male cross-progeny of *cs1* mothers transmitted *cs1* to 100% of their progeny. *cs1* failed to complement *ksr-1(n2526)* for the ability to suppress the Muv phenotype of *let-60(n1046gf)*.

sur-6(cs24) I: Two-factor mapping experiments and complementation tests were performed in the *lin-45(ku112)* background. Of 37 *cs24* homozygotes from *cs24/unc-13* mothers, 1 segregated *unc-13*. *cs24* failed to complement *sur-6(ku123)* for the enhancer phenotype.

sur-2(cs26, cs31) I: Two-factor mapping experiments and complementation tests were performed in the *lin-45(ku112)* background. Of 41 *cs31* homozygotes from *cs31/unc-54* mothers, 3 segregated *unc-54*. *cs26* failed to complement *cs31* and *sur-2(ku9)* for the enhancer phenotype.

lin-25(cs52) V: Three-factor mapping experiments and complementation tests were performed in the *lin-45(ku112)* background. Of 48 *cs52* homozygotes from *cs52/dpy-11 unc-76* mothers, 4 segregated *dpy-11* and 2 segregated *unc-76*. *cs52* failed to complement *lin-25(e1446)* for the enhancer phenotype.

eor-1(cs28, cs40, cs44) IV: In initial outcrosses with *eor-1 lin-45(ku112) dpy-20* strains, >75% of Dpy animals but almost no non-Dpy animals were Egl, placing *eor-1* on chromosome IV. In three-factor mapping experiments, 33/46 Dpy-13 not-Unc-5 recombinants from *cs28/dpy-13 unc-5* mothers segregated *cs28*, placing *eor-1* between these two markers. *mDf8*, *cs40*, and *cs44* failed to complement *cs28* for the lethal and/or enhancer phenotypes. *eor-1* maps in the vicinity of *sur-8*; however, both complementation testing and molecular analysis have verified that *eor-1* is a distinct locus (R. M. Howard and M. V. Sundaram, unpublished results). *eor-1 (cs28)* also complemented *let-277*, *let-279*, *let-280*, *let-281*, *let-282*, and *let-284*.

eor-2(cs7, cs30, cs42, cs47, cs51) X: eor-2 was initially mapped to the X chromosome on the basis of the observation that male progeny of *eor-2* mothers are uncoordinated (Unc) and incapable of mating. Of 58 *cs30* homozygotes from *cs30/unc-3* mothers, 1 segregated *unc-3*. In three-factor mapping experiments, 4/4 Eor-2 not-Unc-3 recombinants from *cs30 unc-3/lin-15* mothers segregated *lin-15*, placing *eor-2* to the left of *unc-3*. *mnDf7*, *cs7*, *cs42*, *cs47*, and *cs51* all failed to complement *eor-2(cs30)* for the Unc and/or lethal phenotypes. *eor-2(cs30)* complemented *let-4* and *syc-2*.

Phenotypic observations: General methods for Nomarski differential interference contrast (DIC) microscopy of live animals were as previously described (Sulston and Horvitz 1977).

Lethal and Egl phenotypes: Two or more hermaphrodites of the indicated genotype were picked singly to plates and allowed to lay eggs for 8–24 hr. Rod-like arrested larvae were counted and removed after 1–2 days. Surviving adults were scored as Egl if they appeared bloated with late-stage eggs or hatching larvae. One to three percent of *eor-1* and *eor-2* mutants die as rod-like young adults, suggesting a late defect in excretory system development or function; these are not included in the larval lethal or Egl categories.

Vul phenotype: The numbers of vulval and nonvulval descendants of P(3-8).p were counted in L4 stage larvae under DIC optics. Animals with <22 vulval descendants and >6 nonvulval descendants were considered Vulvaless. In some *sur-6(cs24)* mutants, but not in other mutants described here, some P(4-8).p cells failed to divide and may have adopted an abnormal fused fate (Sieburth *et al.* 1999).

2 P11.p phenotype: Under DIC optics, P11.p and P12.pa nuclei are distinguishable on the basis of nuclear size, morphology, and position (Jiang and Sternberg 1998). The number of P11.p-like nuclei anterior to the anus was counted in L3 or L4 stage larvae.

Sequencing of mutant alleles: The *ksr-1(cs1)*, *sem-5(cs15)*, and *lin-1(cs50)* lesions were identified by direct sequencing of genomic PCR products, and the *lin-45(ku112)* and *let-341(cs41)* lesions were identified by direct sequencing of RT-PCR products. In each case the entire gene coding region was sequenced (except for *lin-1*, in which only the 3′ half was sequenced) and only a single lesion was identified. *lin-1* was also sequenced from the MH620 strain to verify that the *cs50* lesion was not present in this parental *lin-45(ku112)* strain. All lesions were verified by sequencing at least two independently derived PCR products.

RESULTS

***lin-45* hypomorphs are sensitized to further reductions in Ras signaling:** The *lin-45(ku51)* and *lin-45(ku112)* alleles weakly reduce *lin-45 raf* activity but do not cause overt phenotypes (Table 1A; Sundaram and Han 1995). *ku51* is a missense mutation changing leucine 252 to valine (M. Han, personal communciation). Interestingly, we identified the *ku112* lesion as a missense change affecting the C-terminal conserved 14-3-3 binding site (Figure 2A), suggesting that binding of the chaperone protein 14-3-3 to this site normally promotes LIN-45 activity (see discussion).

Both *lin-45 raf* hypomorphic mutant backgrounds are very sensitive to further reductions in Ras pathway signaling. For example, a putative null allele of *ksr-1*, *n2526*, causes few defects on its own, but causes partially penetrant larval lethal and Egl defects in the *lin-45(ku51)* background and more highly penetrant larval lethal, Egl, and Vul defects in the *lin-45(ku112)* background (Table 1B; Sieburth *et al.* 1999). Furthermore, even a very weak allele of *mpk-1* causes nearly complete lethality in the *lin-45(ku112)* background (Sundaram and Han 1995). These observations suggested that we might identify new positive regulators of Ras signaling by screening for enhancers of *lin-45* hypomorphic defects.

Genetic screens for enhancers of *lin-45* hypomorphic alleles: We screened for recessive mutations that cause a "bag of rods" phenotype in the *lin-45(ku51)* or *lin-45 (ku112)* mutant backgrounds (materials and methods). Homozygous *lin-45* hermaphrodites were mutagenized with EMS, and F_1 progeny, which were potentially heterozygous for an enhancer mutation, were placed on individual petri plates. F_2 progeny were screened for retention of eggs and the presence of rod-like arrested larvae inside the body cavity. Note that since this screen required that F_2 animals survive to adulthood and give dead F_3 progeny, it would not identify mutations that cause significant F_2 lethality or sterility; we

TABLE 1

***lin-45* hypomorphic mutants are sensitive to further reductions in Ras pathway activity**

	Genotype[a]	% rod-like lethal (*n*)	% Egl (*n*)	% Vul (*n*)	Average no. VPCs induced (*n*)	% 2 P11.p (*n*)
A	+	0	<1	0	3.0	0
	lin-45(ku51)	<1 (238)	<1 (238)	0 (28)	3.0 (28)	0 (30)
	lin-45(ku112)	<1 (198)[b]	3 (198)[b]	0 (24)[b]	3.0 (24)[b]	2 (104)
B	*ksr-1(n2526)*	2 (274)	2 (269)	1 (68)	2.97 (68)	0 (23)
	lin-45(ku51); ksr-1(n2526)	13 (221)	20 (193)	0 (33)	3.0 (33)	0 (19)
	lin-45(ku112); ksr-1(n2526)	56 (185)	100 (84)	70 (23)	2.1 (23)	0 (30)
C	*ksr-1(cs1)*	14 (111)	4 (96)	0 (39)	3.0 (39)	0 (23)
	lin-45(ku51); ksr-1(cs1)	64 (127)	91 (46)	43 (37)	2.04 (37)	0 (25)
	lin-45(ku112); ksr-1(cs1)	100 (many)[c]	ND	ND	ND	ND

n, number of animals scored; ND, not determined.

[a] All *lin-45* chromosomes were marked with *dpy-20*. *ksr-1(n2526)* is a putative null allele and encodes a truncated protein lacking the kinase-like domain (Kornfeld *et al.* 1995). *ksr-1(cs1)* was identified as an enhancer of *lin-45(ku51)* (see text and materials and methods).

[b] Data from Sundaram and Han (1995).

[c] Animals of this genotype could be obtained from heterozygous parents, but their progeny were 100% lethal.

predicted that most mutations in core Ras pathway genes would fall into this category and thus be avoided. From a total of 1316 *ku51* and 7254 *ku112* F_1 animals, we identified 26 bag of rods candidates. Outcrossing revealed that the new mutations were of two types: those that cause strong Egl defects on their own and cause more severe or additional defects in combination with the *lin-45 raf* allele (11 alleles) and those that cause weak or no defects on their own, but cause strong Egl and lethal phenotypes in combination with the *lin-45 raf* allele (15 alleles, 1 of which was subsequently lost; see materials and methods).

Mutations of the first type include alleles of the previously described genes *sur-2* (Singh and Han 1995) and *lin-25* (Tuck and Greenwald 1995; materials and methods); their enhancer properties reveal that these genes positively regulate Ras signaling in tissues other than the vulva, as recently reported by Nilsson *et al.* (2000). These mutations have not been further characterized. Additional mutations of the first type are *sem-5(cs15)* and *let-341(cs41ts)*, which are described below. Mutations of the second type affect five previously described genes (*lin-1*, *ksr-1*, *sur-6*, *sur-2*, *lin-25*) and two new genes (*eor-1*, *eor-2*).

Phenotypes analyzed in the mutant strains: Each new mutation of interest was scored for phenotypic effects in single mutants and in double mutants with *lin-45(ku112)* (Table 2). We scored four phenotypes that are commonly associated with reduced Ras signaling efficiency.

1. *Rod-like larval lethality*: Ras pathway loss-of-function mutants die during early larval development with a distinctive fluid-filled, rod-like appearance (Figure 3, A and B). Mosaic analysis of *let-60 ras* suggested that this lethality is caused by a failure of the excretory duct cell to differentiate properly (Yochem *et al.* 1997), although mosaic analysis of *let-23* suggested that rod-like lethality may also result from distinct excretory system defects (Koga and Ohshima 1995).
2. *Egg-laying (Egl) defects*: Ras pathway loss-of-function mutants are often Egl (Figure 3, C and D). A lack of vulval cells (see below), abnormalities in sex myoblast migration (Sundaram *et al.* 1996), gonadal differentiation (Chang *et al.* 1999), or vulval cell differentiation (Wang and Sternberg 2000) may contribute to this phenotype.
3. *Vulvaless (Vul) defects*: The Ras pathway is required for the vulval precursor cells P5.p, P6.p, and P7.p to adopt vulval fates (Sternberg and Han 1998). In Ras pathway loss-of-function mutants, one or more of these cells often adopts a nonvulval fate, resulting in an incomplete or absent vulva (Figure 3, E and F).
4. *2 P11.p defects*: The Ras pathway is required for the ventral ectodermal blast cell P12 to adopt a fate different from its neighbor P11 (Jiang and Sternberg 1998). In Ras pathway loss-of-function mutants, P12 often adopts a P11 fate, resulting in two P11.p-like descendants anterior to the anus (Figure 3, G and H).

Mutations in core Ras pathway genes: Our enhancer screen identifed only three mutations in core Ras pathway components, and all are unusual and informative alleles.

sem-5(cs15) X: cs15 is an allele of *sem-5*, which encodes a Grb2-like SH3-SH2-SH3 domain adaptor protein that functions to connect LET-23 receptor tyrosine kinase signaling to LET-60 RAS activation (Clark *et al.* 1992). *cs15* appears to partially reduce *sem-5* function, since it causes rod-like lethal, Egl, Vul, and 2 P11.p defects similar to (but less penetrant than) those seen in strong *sem-5* mutants (Table 2A). However, *cs15* also appears to increase signaling activity, since unlike strong *sem-5*

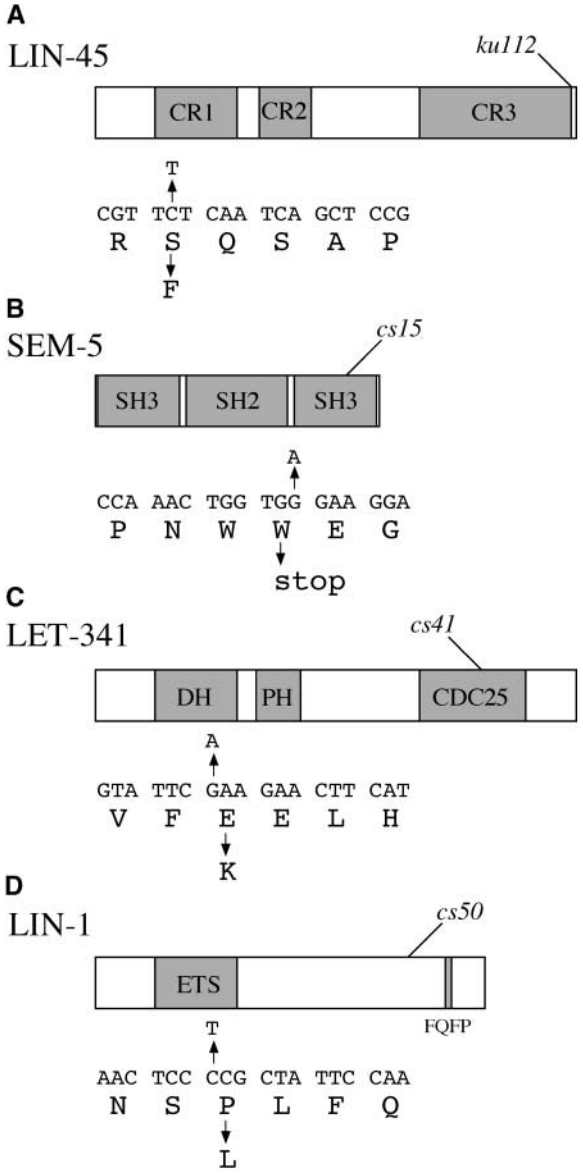

FIGURE 2.—Molecular lesions associated with the *lin-45 (ku112)*, *sem-5(cs15)*, *let-341(cs41ts)*, and *lin-1(cs50)* alleles. Schematic drawings represent each protein, with the general positions of each amino acid substitution marked by the allele name. Below each drawing the specific nucleotide and amino acid substitutions are shown above and below the wild-type sequences, respectively. (A) *lin-45(ku112)* (S754F) affects the C-terminal 14-3-3 binding site. CR1, CR2, and CR3 are conserved regions found in all Raf family members (MORRISON and CUTLER 1997). (B) *sem-5(cs15)* (W192STOP) truncates the protein within the second SH3 domain. (C) *let-341(cs41ts)* (E980K) affects the CDC25 Ras GEF homology domain. DH, Dbl homology domain; PH, pleckstrin homology domain. (D) *lin-1(cs50)* (P316L) affects a minimal consensus mitogen-activated protein kinase phosphorylation site. FQFP, ERK docking site (JACOBS *et al.* 1999).

alleles, *cs15* causes a synthetic Muv phenotype in a *gap-1* [*G*TPase-*a*ctivating *p*rotein (HAJNAL *et al.* 1997)] mutant background (Table 3A). *cs15* also dominantly enhances the activated *let-60 ras* Muv phenotype (Table 3B).

To understand the molecular basis for this unusual genetic behavior, we sequenced the *cs15* allele and identified its lesion. *cs15* is a nonsense mutation predicted to truncate the SEM-5 protein within the second SH3 domain (Figure 2B). This finding suggests that the C-terminal SH3 domain of SEM-5 has an inhibitory or negative signaling function.

let-341(cs41ts) V: cs41 is an allele of *let-341*, which encodes a Sos-related guanine nucleotide exchange factor that acts upstream of *let-60 ras* (JOHNSEN and BAILLIE 1991; CHANG *et al.* 2000). Previously described *let-341* mutants are 100% embryonic or larval lethal. *cs41* is temperature sensitive such that homozygotes appear essentially wild type (but enhance *lin-45(ku112)* defects) at 20°, but have strong rod-like lethal, Egl, Vul, and 2 P11.p defects at 25° (Table 2B). These pleiotropic defects of *let-341(cs41)* animals, and the ability of an activated *let-60 ras* allele to suppress these defects (Table 2B), support a role for Sos in many different Ras-dependent processes in *C. elegans*.

We sequenced the *cs41* allele and found that it contains a missense mutation within the CDC25-like Ras GEF domain (Figure 2C). The affected residue is not well conserved among different Sos family members, but it is located near the tip of the helical hairpin that catalyzes guanine nucleotide exchange on Ras (BORIACK-SJODIN *et al.* 1998).

lin-1(cs50) IV: cs50 is an allele of *lin-1*, which encodes an Ets domain transcription factor that negatively regulates Ras signaling (BEITEL *et al.* 1995). *lin-1* null mutants are Muv. In contrast, *cs50* has no discernible phenotype on its own but strongly enhances the *lin-45(ku112)* rod-like lethal, Vul, and 2 P11.p phenotypes (Table 2C). Since *cs50* has opposite phenotypic effects from those expected for a loss-of-function mutation and is a semidominant enhancer of *lin-45(ku112)*, *cs50* appears to be a weak gain-of-function allele of *lin-1*. We identified the *cs50* lesion as a missense mutation that changes proline 316 of a minimal ERK phosphorylation site (S/T-P) to a leucine (Figure 2D). This suggests that serine 315 may be a target for phosphorylation and negative regulation by MPK-1.

Mutations in positive regulatory genes: Our enhancer screen also identified alleles of four known positive regulatory genes and two new genes.

ksr-1(cs1) X: cs1 is an allele of *ksr-1*, which encodes a kinase-like protein that promotes signaling at a step between Ras and Raf (KORNFELD *et al.* 1995; SUNDARAM and HAN 1995; SIEBURTH *et al.* 1999). *cs1* causes weak Egl and larval lethal phenotypes on its own, strong Egl, Vul, and rod-like lethal phenotypes in the *lin-45(ku51)* background (where it was recovered), and complete lethality in the *lin-45(ku112)* background (Table 1C). Notably, *ksr-1(cs1)* is a stronger enhancer than the putative null allele *ksr-1(n2526)* (Table 1), indicating that this allele has a dominant-negative character. The *cs1* lesion changes arginine 531 to histidine (MATERIALS

TABLE 2

Enhancers of *lin-45 raf*

	Genotype[a]	% rod-like lethal (*n*)	% Egl (*n*)	% Vul (*n*)	Average no. VPCs induced (*n*)	% 2 P11.p (*n*)
A	*sem-5(cs15)*	16 (50)	76 (42)	59 (17)	1.89 (17)	12 (17)
	lin-45; sem-5(cs15)	62 (65)	96 (25)	92 (12)	0.99 (12)	58 (12)
B	*let-341(cs41),* 20°	0 (145)	0 (145)	0 (24)	3.0 (24)	4 (24)
	lin-45; let-341(cs41), 20°	51 (107)	27 (52)	12 (24)	2.91 (24)	8 (24)
	let-341(cs41), 25°	99 (158)	100 (28)	61 (18)	1.86 (18)	67 (18)
	let-60(n1046); let-341(cs41), 25°	0 (58)	ND	0 (20)	3.06 (20)	0 (20)
C	*lin-1(cs50)*	0 (192)	0 (192)	0 (24)	3.0 (24)	0 (23)
	lin-1(cs50) lin-45	70 (153)	91 (46)	32 (19)	2.55 (19)	48 (25)
	lin-1(cs50) lin-45/ + lin-45	ND	9 (28)	ND	ND	ND
D	*sur-6(cs24)*	0 (195)[b]	4 (137)	2 (48)[b]	2.97 (48)[b]	0 (21)
	sur-6(cs24); lin-45	80 (360)[b]	95 (56)	87 (24)[b]	1.5 (24)[b]	23 (31)
E	*sur-2(cs26)*	0 (126)	17 (126)	29 (24)	2.76 (24)	0 (25)
	sur-2(cs26); lin-45	31 (128)	93 (88)	93 (28)	2.04 (28)	0 (30)
	sur-2(cs31)	0 (105)	4 (105)	2 (48)	2.98 (48)	0 (27)
	sur-2(cs31); lin-45	30 (187)	79 (130)	38 (45)	2.73 (45)	2 (40)
F	*lin-25(cs52)*	0 (244)	2 (243)	5 (20)	2.98 (20)	0 (36)
	lin-45; lin-25(cs52)	12 (309)	59 (181)	62 (21)	2.52 (21)	9 (67)
G	*eor-1(cs28)*	7 (109)	11 (101)	0 (46)	3.0 (46)	21 (52)
	eor-1(cs28)/mDf8	21 (383)[c]	25 (115)	0 (30)	3.0 (30)	6 (51)
	eor-1(cs28) lin-45	73 (455)	78 (122)	7 (28)	2.97 (28)	85 (20)
	eor-1(cs40)	17 (167)	14 (138)	0 (21)	3.0 (21)	22 (23)
	eor-1(cs40) lin-45	63 (336)	84 (116)	5 (20)	2.97 (20)	81 (26)
	eor-1(cs44)	13 (342)	7 (290)	0 (20)	3.0 (20)	14 (22)
	eor-1(cs44) lin-45	70 (486)	88 (137)	35 (17)	2.7 (17)	88 (26)
H	*eor-2(cs30)*	7 (257)	10 (240)	0 (30)	3.0 (30)	13 (24)
	eor-2(cs30)/mnDf7	11 (331)[c]	ND	0 (13)	3.0 (13)	ND
	lin-45; eor-2(cs30)	78 (95)	76 (21)	4 (28)	2.97 (28)	79 (19)
	eor-2(cs7)	17 (166)	22 (133)	0 (20)	3.0 (20)	13 (24)
	lin-45; eor-2(cs7)	61 (269)	95 (105)	14 (21)	2.88 (21)	91 (22)
	eor-2(cs42)	4 (106)	6 (102)	0 (33)	3.0 (33)	12 (33)
	lin-45; eor-2(cs42)	25 (145)	63 (106)	0 (18)	3.0 (18)	94 (18)
	eor-2(cs47)	19 (213)	26 (171)	0 (20)	3.0 (20)	10 (21)
	lin-45; eor-2(cs47)	67 (368)	91 (100)	0 (20)	3.0 (20)	79 (24)
	eor-2(cs51)	9 (322)	23 (285)	0 (26)	3.0 (26)	22 (27)
	lin-45; eor-2(cs51)	70 (166)	71 (49)	10 (20)	2.94 (20)	79 (29)

n, number of animals scored; ND, not determined.

[a] The *lin-45* allele used was *ku112*. The *lin-45* chromosome was marked with *dpy-20* in double-mutant strains with *sur-2(cs26)* and *sur-2(cs31)*.

[b] Sieburth *et al.* (1999).

[c] Rod-like larval lethality was scored in total broods of mothers of the indicated genotype.

and methods) and is identical to the previously described *ksr-1(ku68)* lesion (Sundaram and Han 1995).

sur-6(cs24) I: cs24 is a partial loss-of-function allele of *sur-6*, which encodes a B regulatory subunit of protein phosphatase 2A that promotes signaling at a step between Ras and Raf (Sieburth *et al.* 1999). A partial genetic characterization of *cs24* has been described elsewhere (Sieburth *et al.* 1999). *cs24* causes very weak Egl, Vul, and Unc phenotypes on its own, but causes strong rod-like lethal, Egl, and Vul phenotypes and weak Unc and 2 P11.p phenotypes in the *lin-45(ku112)* background (Table 2D; Figure 3F).

sur-2(cs26, cs31) I: cs26 and *cs31* are alleles of *sur-2*, which encodes a possible component of the transcriptional Mediator/Srb complex and functions downstream of *mpk-1* during vulval induction (Singh and Han 1995; Lackner and Kim 1998; Boyer *et al.* 1999). Most *sur-2* alleles cause a strong Vul phenotype, but few other defects. *cs26* and *cs31* cause only weak Egl and Vul phenotypes (Table 2E) and thus appear to be hypomorphic. *cs26* and *cs31* cause strong Egl, Vul, and rod-like lethal phenotypes in the *lin-45(ku112)* background (Table 2E).

lin-25(cs52) V: cs52 is an allele of *lin-25*, which encodes

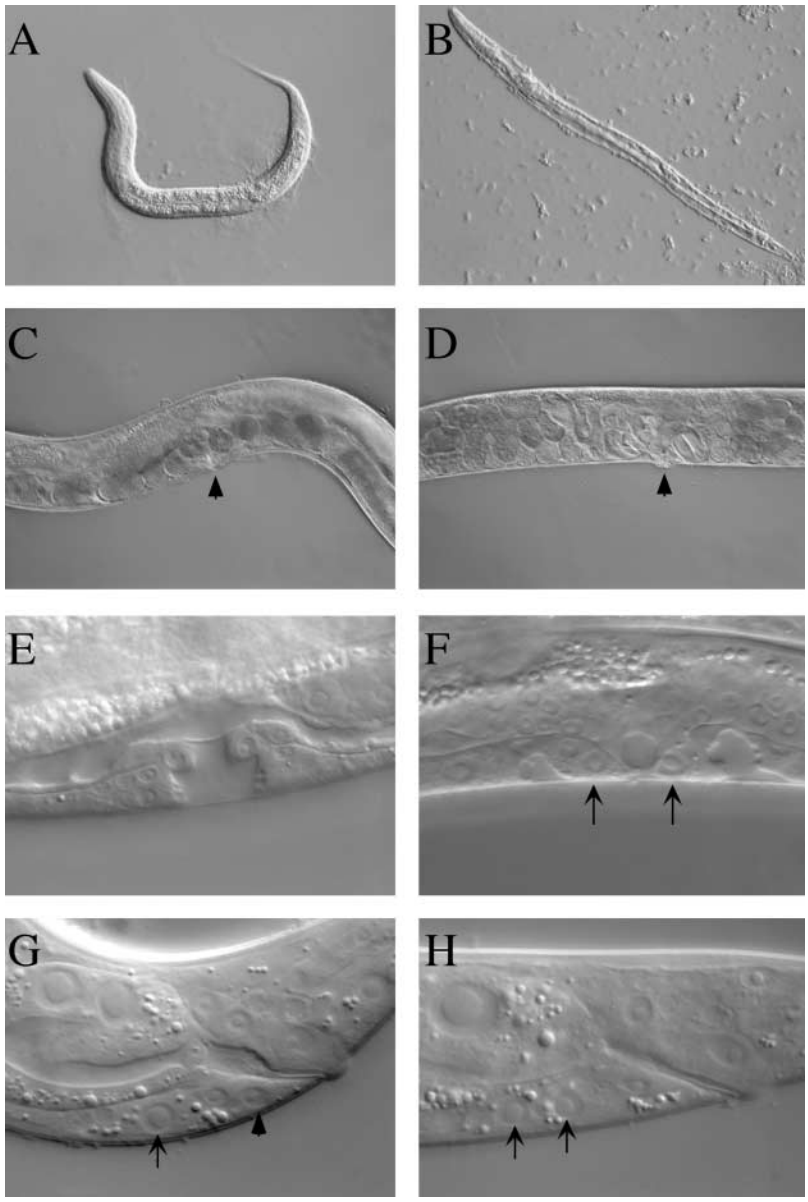

FIGURE 3.—*let-60 ras*-like lethal, Egl, Vul, and 2 P11.p phenotypes seen in enhancer mutants. (A) Wild-type larva. (B) *eor-1(cs28) lin-45(ku112)* rod-like larva. (C) Wild-type adult hermaphrodite; arrowhead indicates vulva. (D) *eor-1(cs28) lin-45 (ku112)* adult bloated with late-stage embryos and hatching larvae; arrowhead indicates normal vulva. (E) Wild-type L4 larva showing normal vulval invagination. (F) *sur-6(cs24); lin-45(ku112)* L4 larva in which P5.pp and P6.pp (arrows) failed to adopt vulval cell fates. (G) Wild-type larva with one P11.p cell (arrow) and one P12.pa cell (arrowhead). (H) *eor-1(cs28) lin-45(ku112)* larva with 2 P11.p-like cells (arrows).

a novel protein thought to function with SUR-2 (TUCK and GREENWALD 1995; NILSSON *et al.* 1998). Most *lin-25* alleles cause a strong Vul phenotype, but few other defects. *cs52* causes only weak Egl and Vul phenotypes (Table 2F) and thus appears to be hypomorphic. *cs52* causes strong Egl, Vul, and rod-like lethal phenotypes and a weak 2 P11.p phenotype in the *lin-45(ku112)* background (Table 2F).

eor-1(cs28, cs40, cs44) IV: These three alleles define a new locus (MATERIALS AND METHODS), which we named *eor-1*. Each allele causes similar weak Unc, Egl, rod-like lethal, and 2 P11.p phenotypes on its own, and weak Unc and Vul but strong Egl, lethal, and 2 P11.p phenotypes in the *lin-45(ku112)* background (Table 2G; Figure 3, B, D, and H). The basis for the Egl phenotype is unknown, as vulval development appears normal in most animals. *eor-1(cs28)/mDf8* animals resemble *eor-1* homozygotes (Table 2G); therefore, the *eor-1* mutations appear to be loss-of-function alleles.

eor-2(cs7, cs30, cs42, cs47, cs51) X: These five alleles behave identically to the *eor-1* mutations described above, but were mapped to a distinct locus (MATERIALS AND METHODS), which we named *eor-2*. Each allele causes similar weak Unc, Egl, rod-like lethal, and 2 P11.p phenotypes on its own, and weak Unc and Vul but strong Egl, lethal, and 2 P11.p phenotypes in the *lin-45(ku112)* background (Table 2H). The basis for the Egl phenotype is unknown as vulval development appears normal in most animals. *eor-2(cs30)/mnDf7* animals resemble *eor-2* homozygotes (Table 2H); therefore, the *eor-2* mutations appear to be loss-of-function alleles.

DISCUSSION

Over the last decade, experiments in multiple systems have elucidated a highly conserved signal transduction pathway involving receptor tyrosine kinases, the Ras

TABLE 3

***sem-5(cs15)* causes a Muv phenotype in sensitized genetic backgrounds**

	Genotype[a]	% Muv	Average no. VPCs induced	(*n*)
A	*sem-5(cs15)*	0	1.89	(17)
	gap-1	0	3.0	(20)
	gap-1 sem-5(cs15)	84	3.96	(19)
	gap-1 sem-5(n2019)	0	ND	Hajnal *et al.* (1997)
B	*sem-5(cs15)/+*	0	ND	(64)
	let-60(gf)/+	2	ND	(47)
	let-60(gf)/+; sem-5(cs15)/+	86	ND	(50)

n, number of animals scored; ND, not determined.

[a] Alleles used were *gap-1(ga133)* and *let-60(n1046gf)*. For *let-60(gf)/+* experiments, *let-60(gf)/dpy-20* males were mated to *dpy-20* or *dpy-20; sem-5(cs15)* hermaphrodites, and non-Dpy cross-progeny were scored for multiple protrusions by dissecting microscope.

GTPase, and the Raf/MEK/ERK kinase cassette. However, many questions remain about how the Raf/MEK/ERK cassette is initially activated, how the strength and duration of signaling are controlled, what key targets ERK phosphorylates, and what other Ras-dependent or Ras-independent factors cooperate with ERK to affect downstream gene expression and specific cellular behaviors. Enhancers of *lin-45 raf*, in principle, could define genes involved in any of these regulatory processes. Our screen identified mutations in three core components of the Ras pathway (*sem-5*, *let-341*, and *lin-1*), two positive regulators that act at a step between Ras and Raf (*ksr-1* and *sur-6*), two positive regulators that act downstream or in parallel to *mpk-1* ERK (*sur-2* and *lin-25*), and two previously uncharacterized positive regulators (*eor-1* and *eor-2*; Figure 1). Although *eor-1* and *eor-2* also have roles in Ras-independent developmental events (M. Herman and M. Hengartner, personal communication), the spectrum of mutations we identified argues strongly for the specificity of our enhancer screen and therefore for a close involvement of *eor-1* and *eor-2* in Ras signaling.

Unusual alleles of *lin-45*, *sem-5*, *let-341*, and *lin-1*: Since our primary goal was to identify new regulators of Ras signaling, a positive aspect of our enhancer screen was that it selectively identified mutations in positive regulatory genes, while for the most part avoided mutations in core components of the Ras pathway. This is probably due to the fact that most alleles of core pathway genes would cause strong F_2 lethality and/or sterility in the *lin-45* hypomorphic mutant backgrounds. However, we did identify single alleles of three core components, *sem-5* Grb2, *let-341* Sos, and *lin-1* Ets, and these alleles pinpoint domains or residues likely to play important roles in regulating these components. The *lin-45 raf* allele that we used for most of our screens also contains an interesting lesion.

lin-45(ku112) is a missense mutation in the C-terminal 14-3-3 binding site of LIN-45 RAF. 14-3-3 is a chaperone protein that binds preferentially to the consensus sequence RSXSXP (Muslin *et al.* 1996). This sequence is found near the C terminus of all Raf family members, and binding of 14-3-3 to this site positively regulates mammalian B-Raf activity (MacNicol *et al.* 2000). The hypomorphic nature of *lin-45(ku112)* (which instead has the sequence RFXSXP) suggests that 14-3-3 binding to this site likely positively regulates LIN-45 Raf activity in *C. elegans* as well.

sem-5(cs15) truncates the second SH3 domain of the SEM-5 adaptor. While the first SH3 domain of SEM-5/Grb2 is primarily responsible for interactions with Sos (Sastry *et al.* 1995), the role of the second SH3 domain is less clear. Our finding that *cs15* can increase Ras signaling suggests that the second SH3 domain has an inhibitory or negative signaling function. Such a negative function for Grb2 family members has been proposed recently on the basis of physical interactions between Grb2 proteins and negative regulators such as the adaptor protein Disabled (Le and Simon 1998; Xu *et al.* 1998; Zhou and Hsieh 2001) or the tyrosine kinase ARK-1 (Hopper *et al.* 2000). However, genetic evidence for a Grb2 negative function has until now been limited to the case of one Ras-independent RTK-mediated process (Hopper *et al.* 2000). The *sem-5(cs15)* allele will be a valuable tool for investigating further the negative function of SEM-5/Grb2, which our data suggest may be of widespread importance.

let-341(cs41ts) contains a missense mutation in the helical hairpin of the Ras GEF domain of Sos and causes a spectrum of defects consistent with reduced Ras signaling. Therefore, *cs41* is likely to specifically affect the ability of LET-341 Sos to catalyze guanine nucleotide exchange on LET-60 RAS and may not affect Dbl-domain-mediated exchange activity toward Rho family GTPases (*e.g.*, Nimnual *et al.* 1998). This temperature-sensitive allele will be very useful in dissecting the contributions of LET-341 Sos to different Ras-mediated processes.

lin-1(cs50) is an apparent gain-of-function allele and contains a missense mutation that changes the proline of a consensus ERK phosphorylation site to a leucine. All previously described *lin-1* gain-of-function mutations appear to disrupt the C-terminal ERK docking site of LIN-1, suggesting that these mutations are able to escape negative regulation by MPK-1/ERK (Jacobs *et al.* 1998, 1999). However, LIN-1 has 18 potential ERK phosphorylation sites (Beitel *et al.* 1995), and it is not yet known which of these sites is important for LIN-1 regulation. On the basis of the *cs50* lesion and its weak gain-of-function effects, we hypothesize that Ser315 is one of multiple MPK-1/ERK phosphorylation sites required to downregulate LIN-1.

Genes that act between Ras and Raf: Our enhancer screen identified single non-null alleles of *ksr-1* and *sur-6*, two genes that positively regulate Ras signaling at a step between Ras and Raf (Figure 1). These mutations cause only very mild *let-60 ras*-like defects, but strongly enhance the rod-like lethal and Vul defects of *lin-45(ku112)* mutants. *sur-6(cs24)*, unlike *ksr-1* mutations, also enhances the *lin-45(ku112)* 2 P11.p defect.

KSR is a conserved Raf-related protein (Kornfeld *et al.* 1995; Sundaram and Han 1995; Therrien *et al.* 1995) that binds to MEK (Denouel-Galy *et al.* 1997; Yu *et al.* 1997) and in mammalian cells is found in a large protein complex containing Raf, MEK, ERK, and a number of other proteins (Stewart *et al.* 1999). KSR has therefore been proposed to be a scaffold protein that assembles Raf/MEK/ERK signaling complexes and/or recruits other regulators into such complexes (Morrison 2001). *C. elegans* has two partially redundant *ksr* genes, *ksr-1* and *ksr-2* (Ohmachi *et al.* 2002). The *ksr-1(cs1)* allele identified in our *lin-45(ku51)* enhancer screen is a missense allele encoding R531H. The corresponding variant of murine KSR (R615H) is severely compromised for MEK binding, but still interacts with many other proteins in the KSR complex (Stewart *et al.* 1999), perhaps explaining the dominant-negative behavior of this allele. A propensity for *ksr-1* missense alleles to be dominant negative (Sundaram and Han 1995) and for *ksr-2* mutants to be sterile (Ohmachi *et al.* 2002) may explain our failure to recover any *ksr* alleles in the *lin-45(ku112)* background.

SUR-6 is a PR55 family B regulatory subunit for protein phosphatase 2A (PP2A) and may direct the PP2A catalytic core to a particular Ras pathway substrate such as LIN-45 RAF or KSR-1 (Sieburth *et al.* 1999). The *sur-6(cs24)* allele identified in our screen is clearly non-null, since RNA-mediated interference indicates that *sur-6* is an essential gene (Sieburth *et al.* 1999; Fraser *et al.* 2000; Piano *et al.* 2000). However, even though *cs24* is nearly wild type with respect to viability, it behaves as a strong loss-of-function allele with respect to its *ras*-like phenotypes (Sieburth *et al.* 1999). *cs24* is a missense mutation affecting one of several highly conserved WD repeats (Neer *et al.* 1994; Sieburth *et al.* 1999), and we propose that it could specifically compromise an interaction between SUR-6 and a Ras pathway component or regulator.

Genes that are required primarily for vulval development: Our screen identified multiple alleles of *sur-2* and *lin-25*, two genes that act downstream of *mpk-1* to promote vulval fates (Figure 1). *sur-2* encodes a novel but conserved protein that interacts with components of the human Mediator/Srb complex (Singh and Han 1995; Boyer *et al.* 1999). *lin-25* also encodes a novel protein (Tuck and Greenwald 1995) and is thought to function closely with *sur-2* (Nilsson *et al.* 1998, 2000). Since the Mediator/Srb complex associates with RNA polymerase II and recruits it to certain promoters (Rachez and Freedman 2001), these genes are thought to positively regulate Ras target gene transcription. We isolated several strong *sur-2* and *lin-25* alleles that cause a Vul phenotype like that of previously described mutants, and several hypomorphic alleles that cause few phenotypes on their own but enhance *lin-45(ku112)* lethal, Egl, and Vul defects. Our results reinforce the findings of Nilsson *et al.* (2000), who showed that *sur-2* and *lin-25* function in several Ras-mediated processes, although they are required primarily for vulval development.

Genes that are required primarily for Ras-mediated processes other than vulval development: Our screen identified multiple loss-of-function alleles of two genes, *eor-1* and *eor-2*, that have a relatively weak role during vulval development but appear to positively regulate Ras-mediated signaling in multiple other tissues. *eor-1* and *eor-2* mutations cause a similar spectrum of weakly penetrant rod-like lethal, Egl, and 2 P11.p defects, and these defects are dramatically enhanced in the *lin-45 (ku112)* mutant background. It is interesting to note that this spectrum of defects is somewhat reciprocal to those caused by *sur-2* or *lin-25* mutations, suggesting that different Ras-mediated developmental events have different requirements for *eor-1* and *eor-2* activity *vs. sur-2* and *lin-25* activity.

Our recent studies have shown that *eor-1* and *eor-2* function downstream or in parallel to *mpk-1* and encode nuclear proteins that likely act at the level of transcriptional regulation (R. M. Howard and M. V. Sundaram, unpublished results; Figure 1). Like *sur-2* and *lin-25*, *eor-1* and *eor-2* could be downstream targets of the Ras pathway or could cooperate with the Ras pathway to promote certain cellular outcomes. Further studies of *eor-1* and *eor-2* should provide insight into the important question of how Ras signaling controls different downstream transcriptional responses.

We thank Yuming Han for isolating the *lin-45(ku51)* allele and Min Han for sharing unpublished data and for support during the design of this screen. We also thank Michael Hengartner and Michael Herman for sharing unpublished data, the Caenorhabditis Genetics Center for worm strains, and Elizabeth Bucher and members of our laboratory for helpful comments and advice. This work was supported by a Penn-Hughes award and National Institutes of Health grant 1R01GM58540-01A1 to M.V.S. C.E.R. is a post-doctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research. R.M.H. is a predoctoral trainee supported by the National Institutes of Health Training Program in Developmental Biology grant 5-T32-HD-07516.

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Communicating editor: P. Anderson