Caenorhabditis elegans lin-45 raf Is Essential for Larval Viability, Fertility and the Induction of Vulval Cell Fates

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ABSTRACT

The protein kinase Raf is an important signaling protein. Raf activation is initiated by an interaction with GTP-bound Ras, and Raf functions in signal transmission by phosphorylating and activating a mitogen-activated protein (MAP) kinase kinase named MEK. We identified 13 mutations in the *Caenorhabditis elegans lin-45 raf* gene by screening for hermaphrodites with abnormal vulval formation or germline function. Weak, intermediate, and strong loss-of-function or null mutations were isolated. The phenotype caused by the most severe mutations demonstrates that *lin-45* is essential for larval viability, fertility, and the induction of vulval cell fates. The *lin-45(null)* phenotype is similar to the *mek-2(null)* and *mpk-1(null)* phenotypes, indicating that LIN-45, MEK-2, and MPK-1 ERK MAP kinase function in a predominantly linear signaling pathway. The *lin-45* alleles include three missense mutations that affect the Ras-binding domain, three missense mutations that affect the protein kinase domain, two missense mutations that affect the C-terminal 14-3-3 binding domain, three nonsense mutations, and one small deletion. The analysis of the missense mutations indicates that Ras binding, 14-3-3-binding, and protein kinase activity are necessary for full Raf function and suggests that a 14-3-3 protein positively regulates Raf-mediated signaling during *C. elegans* development.

A small number of distinct signaling pathways are used reiteratively during animal development to control many different cell fate choices. These pathways have been conserved during evolution, and the current understanding of the identity and function of the signaling proteins that comprise these pathways is based on analyses of several organisms, including vertebrates, *Caenorhabditis elegans*, and Drosophila. One such pathway consists of seven core signaling proteins (Cantley *et al.* 1991; Dickson and Hafen 1994; Marshall 1994). The pathway is activated by a protein ligand such as epidermal growth factor (EGF). This ligand binds a receptor tyrosine kinase (RTK), resulting in dimerization and autophosphorylation. These phosphotyrosine residues create docking sites for proteins that contain Src homology 2 (SH2) domains, such as the SH2-SH3-SH2 adapter Grb2/SEM-5. This adapter protein interacts with a guanine nucleotide exchange factor such as Son of Sevenless (Sos). Sos activates the small GTPase Ras by catalyzing the conversion of inactive GDP-bound Ras to active, GTP-bound Ras. A crucial effector of Ras is Raf, a protein kinase that phosphorylates and activates a mitogen-activated protein (MAP) kinase kinase named MEK, which phosphorylates and activates extracellular-signal-regulated kinase (ERK), a member of the MAP kinase family. ERK phosphorylates a variety of proteins, including transcription factors, and thus is likely to be an important link between signaling proteins and proteins that mediate particular cell fates (Treisman 1996). In addition to these core signaling proteins, regulatory and effector proteins feed into and out of this pathway at multiple levels.

In the nematode worm *C. elegans*, an RTK/Ras/ERK signaling pathway has been analyzed most extensively during the formation of the hermaphrodite vulva (Horvitz and Sternberg 1991; Greenwald 1997; Kornfeld 1997; Sternberg and Han 1998). In third larval stage hermaphrodites, six ventral epidermal blast cells called P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p (Pn.p cells) lie along the anterior-posterior axis. Each of these Pn.p cells can adopt any of three distinct fates: the primary (1°) vulval cell fate (eight descendants), the secondary (2°) vulval cell fate (seven descendants), or the tertiary (3°) nonvulval cell fate (two descendants). The anchor cell of the somatic gonad signals P6.p to adopt the 1° fate by activating an RTK/Ras/ERK signaling pathway. P6.p signals P5.p and P7.p to adopt the 2° fate by activating *lin-12*, which is similar to the receptor Notch. P3.p, P4.p, and P8.p receive neither signal and adopt the 3° fate. The 22 descendants of P5.p, P6.p, and P7.p generate the vulva, a specialized epidermal structure used for egg laying. Similar pathways also control other cell fate decisions including the differentiation of the excretory duct cell, which is necessary for larval viability, and the

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progression of germ cells through pachytene, which is necessary for hermaphrodite fertility (Church *et al.* 1995; Yochem *et al.* 1997).

The anchor cell and P6.p communicate using a highly conserved signal transduction pathway that includes the *lin-3* ligand, which is similar to EGF; the *let-23* RTK; the *sem-5* adapter protein; *let-341* guanine nucleotide exchange factor; *let-60* Ras; *lin-45* Raf; *mek-2* MEK; *mpk-1* ERK; and *lin-1* ETS transcription factor (Kornfeld 1997; Sternberg and Han 1998). With the exception of *lin-1*, a mutation that reduces the activity of one of these genes causes all six Pn.p cells to adopt the nonvulval 3° fate, resulting in a vulvaless (Vul) phenotype. By contrast, a constitutively active form of one of these genes causes all six Pn.p cells to adopt the 1° or 2° vulval fate, resulting in a multivulva (Muv) phenotype characterized by ectopic patches of vulval tissue. The signaling pathway negatively regulates the LIN-1 ETS transcription factor, so a *lin-1(lf)* mutation causes a Muv phenotype. These phenotypes are dramatic, and thus the extent of vulval induction can serve as an easily visualized readout of Ras pathway activity.

Here we focus on the Raf protein kinase. Raf has been analyzed extensively using purified Raf protein and Raf expressed in vertebrate cultured cells. Prior to ligand stimulation, Raf is catalytically inactive and localized to the cytoplasm in a multiprotein complex. The initial event in Raf activation is the recruitment of Raf to the plasma membrane through a high-affinity interaction between the switch 1 region of activated Ras-GTP and the N-terminal minimal Ras-binding domain of Raf (Vojtek *et al.* 1993; Zhang *et al.* 1993; Stokoe *et al.* 1994; Finney and Herrera 1995; Marais *et al.* 1995). Plasma membrane-localized Raf is then activated by a mechanism that has yet to be fully characterized, but it appears to depend on relieving the interaction between the N-terminal regulatory domain and the C-terminal kinase domain (Stanton *et al.* 1989; Heidecker *et al.* 1990). Activated Raf phosphorylates MEK on two serine residues, which greatly stimulates MEK kinase activity (Alessi *et al.* 1994; Gardner *et al.* 1994; Zheng and Guan 1994). Raf has been reported to interact with several other proteins that may positively or negatively modulate Raf activity. These include 14-3-3, Ksr, Hsp90, Cdc37, and a variety of protein kinases and phosphatases (Morrison and Cutler 1997). The physiological significance of many of these interactions has yet to be fully characterized.

By contrast to the large number of studies of vertebrate Raf, the *C. elegans lin-45 raf* gene has not been analyzed extensively. Han *et al.* (1993) molecularly characterized *lin-45* and demonstrated that *C. elegans* Raf is similar to vertebrate Raf in conserved regions (CR) 1, 2, and 3. A single mutation in the *lin-45* gene, *lin-45(sy96)*, which reduces but does not eliminate the activity of the *lin-45* locus, was identified. The analysis of this mutant allele indicated that Raf is important for RTK/Ras/ERK signaling pathways at multiple times during development (Han *et al.* 1993). Here we present an analysis of 13 additional alleles of *lin-45*, including weak, intermediate, and strong loss-of-function mutations. The strong loss-of-function or null alleles demonstrate that Raf is required for Ras-mediated signaling during larval development, oocyte maturation, and vulval induction. These mutations were analyzed molecularly, and eight are missense mutations that identify functionally significant residues in the Ras-binding domain, the protein kinase domain, and the C-terminal 14-3-3 binding domain. Our results demonstrate the importance of these residues and domains for Raf function and suggest that the interactions of Raf with these binding proteins are important for Raf activity.

MATERIALS AND METHODS

General methods and strains: *C. elegans* strains were cultured as described by Brenner (1974) and grown at 20° unless otherwise noted. The wild-type strain and parent of all mutant strains was N2. The following mutations cause a visible phenotype, were used to mark chromosomes, and are described by Riddle *et al.* (1997): LGI, *sup-11(n403)*; *dpy-5(e61)*; LGIII, *unc-79(e1068)*; *dpy-17(e164)*; and LGIV, *unc-24(e138)*; *unc-5(e53)*; *dpy-20(e1282)*; *bli-6(sc16)*. Standard techniques were used to mutagenize animals and screen for mutants, separate the *lin-45* mutations from the *let-60*, *lin-15*, or *gon-2 unc-29* mutations, backcross newly identified mutations using N2, position mutations on the genetic map, perform complementation tests, and generate double mutants (Brenner 1974).

The following mutations that affect vulval development were used: *lin-15(n309)* is a recessive, strong loss-of-function or null allele caused by an ~13-kb deletion that removes all of *lin-15A* and most of *lin-15B* (Clark *et al.* 1994). *let-60(n1046 G13E)* is a semidominant, gain-of-function allele (Beitel *et al.* 1990). Similar mutations of vertebrate Ras result in oncogenic proteins that have reduced GTPase activity. *lin-1(e1275 R175Stop)* is a recessive, heat-sensitive, partial loss-of-function allele that truncates LIN-1 downstream of the ETS DNA-binding domain (Beitel *et al.* 1995). *lin-31(n1053 Trp57Stop)* is a recessive, strong loss-of-function or null allele (Miller *et al.* 2000). *lin-12(n137 S872P)* is a dominant gain-of-function allele that affects the extracellular domain of the LIN-12 Notch receptor (Greenwald and Seydoux 1990). *mek-2(n2516* E238K*)* and *mek-2(n2678* D213N*)* are recessive, strong loss-of-function or null alleles that alter highly conserved residues in the kinase domain (Kornfeld *et al.* 1995a). *mpk-1(ga117)* is a recessive, strong loss-of-function allele caused by a single-nucleotide change in a splice site upstream of residue 24 that eliminates detectable MPK-1 protein (Lackner and Kim 1998). *lin-45 (sy96)* is a recessive, partial loss-of-function allele caused by a single nucleotide change in a splice site upstream of residue 229 that eliminates most but not all functional *lin-45* mRNA (Han *et al.* 1993). *nDf41* is a deletion of ~1.5 map units of chromosome IV that fails to complement genes positioned to the left (*pat-8*) and right (*dif-1*) of *lin-45* (Riddle *et al.* 1997). The *lin-45* alleles *n1924*, *n1925*, *n2018*, *n2506*, *n2510*, *n2520*, *n2523*, *oz166*, *oz178*, *oz201*, *dx19*, *dx84*, and *dx89* are described here.

Identification of the *lin-45* mutations: We previously described a screen for suppressors of the *let-60(gf)* Muv phenotype (Lackner *et al.* 1994; Kornfeld *et al.* 1995a,b; Jacobs *et al.* 1998; Jakubowski and Kornfeld 1999). In brief, we

mutagenized *let-60(n1046)* hermaphrodites with ethyl methane sulfonate (EMS), placed 2794 F_1 self-progeny on separate petri dishes, and examined F_2 self-progeny for non-Muv animals at 22.5°. We identified 33 independently derived mutations that reduced the penetrance of the Muv phenotype from 93 to <10%, including the *lin-45* alleles *n2506*, *n2510*, *n2520*, and *n2523*. In a related screen that was described previously (Beitel *et al.* 1990), *lin-8(n111); let-60(gf)* hermaphrodites were mutagenized with EMS and non-Muv F_1 self-progeny were picked to separate petri dishes at 25°. Ten extragenic mutations identified in this screen met the criteria described above, including the *lin-45* alleles *n1924* and *n1925*. *lin-45(n2018)* was isolated in a previously described screen (Beitel *et al.* 1990; Clark *et al.* 1992) by mutagenizing *lin-15(n765ts)* hermaphrodites with EMS and examining the progeny of 38,000 F_1 animals for non-Muv animals. The *lin-45* alleles *oz166*, *oz178*, and *oz201* were generated by mutagenizing N2 hermaphrodites with EMS and identified in F_2 clonal screens for mutations that caused sterility. The *lin-45* alleles *dx19*, *dx84*, and *dx89* were generated by irradiating adult hermaphrodites with 310 nm ultraviolet light (12–18 sec of ~25 J/m^2/sec). *dx19* was generated by mutagenizing N2 hermaphrodites and identified in an F_2 clonal screen for gonadal defects. *dx89* was generated by mutagenizing *gon-2(q388) unc-29(e1072)* hermaphrodites and identified in an F_2 clonal screen for enhancement of the *gon-2* gonadal defect. *dx84* was generated by mutagenizing N2 hermaphrodites and identified by screening for deletions in the *lin-45* locus using the outer primers 5′-GACATATTTTGTCAGGTAATCG-3′ and 5′-GTC TAAGTGAAGAACATTCGG-3′ and the inner primers 5′-TCT CAATTATTCAGGAGCTCG-3′ and 5′-GAGTCAATTTTGGA AGAATTATG-3′ according to the method described by Denburg *et al.* (1998).

Genetic mapping and complementation tests: The following genetic mapping and complementation experiments support the conclusion that the identified mutations are alleles of *lin-45*. *n1924*, *n1925*, *n2506*, *n2520*, and *n2523* displayed linkage to *dpy-20* IV (data not shown); three factor-mapping experiments indicated that *n1924* is to the left of *unc-24* IV and *n2506* is positioned between *bli-6* IV and *unc-24* IV (data not shown), an ~0.36 map unit interval that contains *lin-45* (Riddle *et al.* 1997). Each of these five alleles failed to complement the other four alleles for the suppression of the *let-60(gf)* Muv phenotype (data not shown), indicating that they represent a single complementation group. *n2510* displayed linkage to *dpy-20* IV and failed to complement the vulval defect caused by *n2018* and *lin-45(sy96)* (data not shown). *oz166* and *oz178* were mapped between *bli-6* IV and *unc-24* IV (data not shown). *oz166* failed to complement the lethality caused by *n1924*, *n1925*, *n2520*, and *n2506* (Table 2) and the vulval defects caused by *sy96*, *n2018*, and *dx19* (data not shown). *dx19* and *dx89* displayed linkage to chromosome IV (data not shown).

Determination of DNA sequences of *lin-45* alleles: For each of the *lin-45* alleles, genomic DNA was derived from homozygous mutant adult hermaphrodites and was amplified by polymerase chain reaction (PCR) according to Williams *et al.* (1992). *lin-45* contains 12 exons (Han *et al.* 1993). To identify the molecular lesion in the 12 alleles identified in screens for visible phenotypes, the oligonucleotide primers Raf-Fwd-Ex1 (5′-GTCACATCATCTCAAACGCC-3′) and Raf-Rev-Ex4 (5′-CCAGGCAGTCGGGATGCG-3′) were used to amplify a DNA fragment containing 25 bp upstream of exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, and 17 bp of exon 4. Raf-Fwd-Ex3 (5′-ACAAGAATCTATTGAATTATCGG-3′) and Raf-Rev-In4 (5′-CAAATGTTGGGACAACATTGG-3′) were used to amplify a DNA fragment containing 51 bp of intron 3, exon 4, and 41 bp of intron 4. Raf-Fwd-In4 (5′-CTCACCTCTGCTTC AGAAAC-3′) and Raf-Rev-Ex7 (5′-GGGGATTGTCCAAGAGT AGG-3′) were used to amplify a DNA fragment containing 29 bp of intron 4, exon 5, intron 5, exon 6, intron 6, and 104 bp of exon 7. Raf-Fwd-Ex7 (5′-CTCGAATGAATCGTCTTCACC-3′) and Raf-Rev-In8 (5′-TTTGTAGAACTGCCGGTTTGC-3′) were used to amplify a DNA fragment containing 191 bp of exon 7, intron 7, exon 8, and 38 bp of intron 8. The amplified regions of exon 7 overlap by 80 bp. Raf-Fwd-In8 (5′-AAAAACG CTCAAAACTTCTCTC-3′) and Raf-Rev-Ex10 (5′-CAGTATAT CAATGATAGCACCC-3′) were used to amplify a DNA fragment containing 28 bp of intron 8, exon 9, intron 9, and 22 bp of exon 10. Raf-Fwd-Ex9 (5′-TATAGACATATTCATGTTCA AGG-3′) and Raf-Rev-In11 (5′-CCCCATAATAAATCATAGTT CTAC-3′) were used to amplify a DNA fragment containing 45 bp of intron 9, exon 10, intron 10, exon 11, and 31 bp of intron 11. Raf-Fwd-In11 (5′-CATGAATAACTCCACTACACTG-3′) and Raf-Rev-Ex12b (5′-GGTACATATTCGGGGAGAGACGAG-3′) were used to amplify a DNA fragment containing 68 bp of intron 11, exon 12, and 58 bp downstream of exon 12.

We purified PCR-amplified DNA fragments and determined the complete sequences of both strands using the amplification primers and additional primers positioned inside the larger DNA fragments with an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Isolation and molecular characterization of *lin-45 raf* alleles: We identified 12 alleles of *lin-45 raf* by conducting several different genetic screens (see materials and methods). The alleles *n1924*, *n1925*, *n2506*, *n2510*, *n2520*, and *n2523* were identified in screens for mutations that suppressed the Muv phenotype caused by a gain-of-function (gf) mutation that constitutively activates the *let-60 ras* gene. The allele *n2018* was identified in a screen for mutations that suppress the Muv phenotype caused by a *lin-15(lf)* mutation. *lin-15* is a negative regulator of vulval cell fates and appears to act upstream of or parallel to *let-60 ras*. The alleles *oz166*, *oz178*, *oz201*, *dx19*, and *dx89* were isolated in screens for mutations that cause sterility or gonadal defects.

Three approaches were used to demonstrate that these 12 mutations are alleles of *lin-45*. First, genetic mapping experiments were used to position the mutations in the region of chromosome IV that contains *lin-45* (see materials and methods). Second, complementation tests showed that these mutations failed to complement the previously characterized *lin-45(sy96)* allele or other alleles in this collection (see materials and methods). Third, DNA from each mutant was isolated and used to determine the DNA sequence of the entire *lin-45* coding region and the regions of introns close to splice sites on the basis of the gene structure characterized by Han *et al.* (1993). These experiments revealed a nucleotide change that affects the *lin-45* coding region in 11 of these 12 alleles (Figure 1). We did not identify the mutation in the *oz166* allele, indicating that this mutation may be outside the region that was analyzed. A thirteenth allele, *dx84*, was isolated by using PCR to screen for deletions in the *lin-45* locus (see materials and methods).

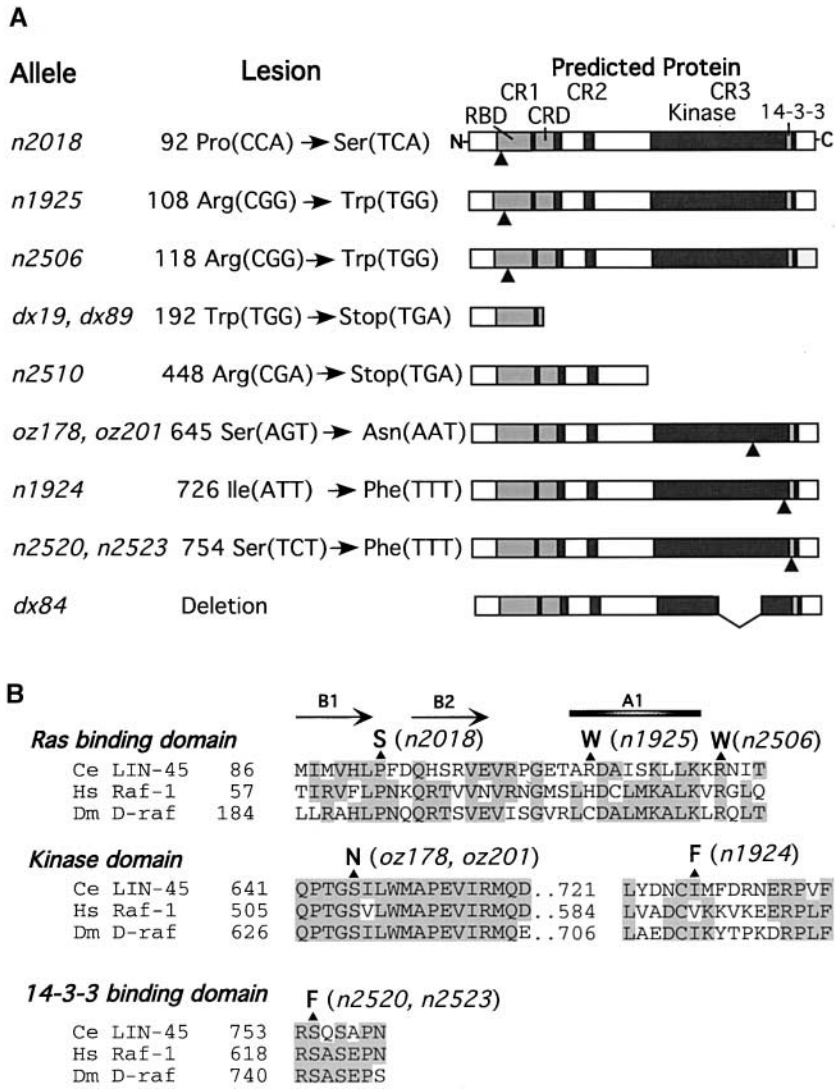

FIGURE 1.—Molecular analysis of *lin-45* alleles. (A) The codon number, wild-type amino acid, and DNA sequence (HAN *et al.* 1993) are followed by the corresponding information for the mutant alleles. Of 11 nucleotide changes, 10 were GC-to-AT transitions, the characteristic mutation caused by ethyl methanesulfonate (COULONDRE and MILLER 1977), the mutagen used to generate all the mutations except *dx19*, *dx89*, and *dx84*. *n1924* had an AT-to-TA transition. *dx84* is an 851-bp deletion that removes 28 bp of intron 10, 412 bp of exon 11, and 411 bp of intron 11. Exon 11 encodes amino acids 601–738, so these residues will be absent from any proteins produced by the *dx84* allele. The predicted LIN-45 proteins are drawn to scale. CR 1, 2, and 3 are darkly shaded. The RBD, CRD, and C-terminal 14-3-3 binding domain (14-3-3) are lightly shaded. Arrowheads indicate the positions of affected amino acids. We detected a single nucleotide change in the *oz166* allele. However, the same nucleotide change was present in the strain prior to mutagenesis, and thus we conclude that this mutation does not cause the *oz166* phenotype. The *oz166* mutation probably lies outside the region that was sequenced. (B) Alignments of the amino acid sequences of *C. elegans* LIN-45 (HAN *et al.* 1993), human Raf-1 (BONNER *et al.* 1986), and *Drosophila melanogaster* D-Raf (NISHIDA *et al.* 1988) for portions of the Ras-binding domain, protein kinase domain, and C-terminal 14-3-3-binding domain; identical residues are shaded. Numbers indicate the position of the first amino acid in each line. Missense changes are shown. Arrows and a bar overlie residues of the Ras-binding domain that form β-sheets (B1 and B2) and an α-helix (A1), respectively (see Figure 2).

Alignments of Raf proteins from many species reveal three highly conserved regions designated CR1, CR2, and CR3. CR1 contains the minimal Ras-binding domain (RBD) and a cysteine-rich domain (CRD; Figure 1A). The alleles *n2018*, *n1925*, and *n2506* contained missense mutations in the Ras-binding domain. *n2018* changes a conserved proline to serine, *n2506* changes a conserved arginine to tryptophan, and *n1925* changes a nonconserved arginine to tryptophan (Figure 1B). The alleles *dx19* and *dx89* contained the same mutation, a nonsense change that is predicted to terminate the LIN-45 protein at residue 192 (Figure 1A). The allele *n2510* contained a nonsense mutation that is predicted to terminate LIN-45 at residue 448 (Figure 1A). These mutant proteins lack the kinase domain. The alleles *oz178*, *oz201*, and *n1924* contained missense mutations in the kinase domain. The *oz178* and *oz201* alleles contained the same mutation and change a highly conserved serine to asparagine (Figure 1B). The *n1924* mutation changes a moderately conserved isoleucine to phenylalanine (Figure 1B). The *dx84* mutation is a deletion that removes all of exon 11 and portions of introns 10 and 11 (Figure 1A). Exon 11 encodes residues 601–738; this region includes many highly conserved residues in the kinase domain. The *n2520* and *n2523* alleles contained the same nucleotide change, a missense mutation that changes the C-terminal 14-3-3 binding domain by converting a highly conserved serine to phenylalanine (Figure 1B).

***lin-45* mutations cause larval lethality, defective vulval development, and sterility and can be arranged in an allelic series:** To determine how these mutations affect the activity of *lin-45* and characterize the role of *lin-45* during development, we analyzed the phenotypes of the mutants. The analysis of *lin-45(sy96)* by HAN *et al.* (1993) showed that a reduction of *lin-45* activity causes partially penetrant larval lethality, sterility, and vulval defects and indicated that *lin-45* is a positive regulator of Ras-mediated signaling. To carefully examine these processes, we placed a single egg on a petri dish and used a dissecting microscope to determine whether the animal died during larval development, whether the adult hermaphro-

TABLE 1

***lin-45* mutations cause larval lethality, sterility, and abnormal vulval formation**

Genotype of parent[a]	Genotype of F_1 progeny[a]	% larval lethal[b]	n[b]	% abnormal vulva[c]	% sterile[c]	n[c]	P5.p–P7.p descendants[d]	n[e]	Classification[f]
Wild type	Wild type	0	200	0	0	200	22 (22)	10	—
n2520/n2520	*n2520/n2520*	0	217	0	0	217	22 (22)	10	W
n1924/n1924	*n1924/n1924*	5	175	0	1	166	ND		W
n1925/n1925	*n1925/n1925*	1	210	1	0	207	ND		W
n2018/n2018	*n2018/n2018*	76	186	24	1	147	ND		I
n2506/n2506	*n2506/n2506*	86	426	93	3	57	18.5 (11–22)	10	I
dx19/+	*dx19/dx19*	13	70	100	100	61	ND		S
dx89/+	*dx89/dx89*	47	57	100	100	30	ND		S
n2510/+	*n2510/n2510*	30	71	100	100	50	ND		S
oz201/+	*oz201/oz201*	55	65	100	100	29	ND		S
dx84/+	*dx84/dx84*	46	52	100	100	28	ND		S
oz166/+	*oz166/oz166*	68	108	100	100	37	6 (6)	10	S
oz166/+	*oz166/+*	0	94	0	0	94	ND		

[a] F_1 self-progeny were analyzed by placing each egg from a parent hermaphrodite on a separate petri dish and observing development with a dissecting microscope. Additional *n2018/n2018* adult hermaphrodites were examined by placing L4 stage larvae on separate petri dishes. The complete genotypes of parent hermaphrodites were as follows: *lin-45(n2520) unc-24*; *lin-45(n1924) unc-24*; *lin-45(n1925) unc-24*; *lin-45(n2018) dpy-20*; *lin-45(n2506) unc-24*; *lin-45(dx19)/unc-24*; *lin-45(dx89)/unc-24 dpy-20*; *lin-45(n2510) unc-24/unc-5 dpy-20*; *lin-45(oz201)/unc-5 dpy-20*; *lin-45(dx84)/unc-24 dpy-20*; and *lin-45(oz166)/unc-5 dpy-20*. If F_1 self-progeny of heterozygous hermaphrodites displayed larval lethality or sterility, they were scored as homozygous *lin-45* mutants. This was usually ~25% of the F_1 self-progeny. F_1 self-progeny of *lin-45(oz166)/unc-5 dpy-20* hermaphrodites that were fertile and segregated UncDpy self-progeny were scored as *oz166/+*.

[b] The percentage of hatched eggs judged to have the indicated F_1 genotype that generated dead larvae; most dead larvae displayed a rigid, rod-like morphology. For strains with homozygous mutant parents, *n* is the total number of hatched eggs analyzed. For experiments with heterozygous parents, *n* is the number of dead larvae plus sterile adults.

[c] The percentage of all adult hermaphrodites judged to have the indicated F_1 genotype that displayed a severe egg-laying defect, no discernible vulva, or a protruding vulva (abnormal vulva) or generated no larval progeny (sterile). For experiments with homozygous mutant parents, *n* is the total number of adults analyzed. For experiments with heterozygous parents, *n* is the number of sterile adults.

[d] The number of nuclei that appeared to be descendants of P5.p, P6.p, and P7.p on the basis of appearance and position in L4 hermaphrodites at the "Christmas tree" stage of vulval development. The number is an average followed by the smallest and largest values observed. ND, not determined.

[e] *n*, number of L4 hermaphrodites examined.

[f] Allele severity was classified as weak (W), intermediate (I), and strong (S).

dite laid eggs normally and appeared to have a normal vulva, and whether the adult hermaphrodite generated live progeny. If a mutation could be maintained as a homozygous strain, then eggs were derived from homozygous mutant hermaphrodites. If a mutation caused a fully penetrant sterile phenotype, then eggs were derived from heterozygous mutant hermaphrodites. *n2520*, *n1924*, and *n1925* caused little or no lethality, gross vulval defects, or sterility (Table 1, lines 2–4). *n2018* and *n2506* caused significant larval lethality (76 and 86%), and many of the surviving adult hermaphrodites had an abnormal vulva (24 and 93%). However, these mutations caused only a low-penetrance sterile phenotype (1 and 3%; Table 1, lines 5 and 6). The mutations *n2510*, *oz166*, *oz178*, *oz201*, *dx19*, *dx84*, and *dx89* caused similar defects; each mutation caused a partially penetrant larval lethal phenotype and fully penetrant vulvaless and sterile phenotypes (Table 1, lines 7–12, and data not shown). Thus, these mutations could not be propagated as homozygous mutants.

The finding that the mutations that cause completely penetrant Vul and sterile phenotypes cause only partially penetrant larval lethality might indicate maternal rescue of the larval lethality in homozygous mutants derived from heterozygous hermaphrodites. This type of maternal rescue has been previously documented for *mek-2(null)* and *mpk-1(null)* mutations (Kornfeld *et al.* 1995a; Lackner and Kim 1998). To investigate this possibility, we generated *trans*-heterozygotes with the *oz166* allele, which caused a completely penetrant sterile phenotype, and *n2520*, *n1924*, *n1925*, and *n2506* (Table 2). *Trans*-heterozygous hermaphrodites generated progeny with three different genotypes that were distinguished using marker mutations (Table 2 legend). Homozygous *oz166* animals derived from *trans*-heterozygous hermaphrodites displayed highly penetrant or completely penetrant larval lethality (85–100%; Table 2, lines 3, 7, 10, and 15). These findings indicate that *oz166* strongly affects the ability of *lin-45* to function during embryonic and/or larval development and the survival of *oz166/oz166* animals derived from an *oz166/+* hermaphrodite is due to maternal rescue. Furthermore, these findings

TABLE 2

Complementation analysis of *lin-45* mutations

Genotype of parent(s)[a]	Genotype of F_1 progeny[a]	% larval lethal	*n*	% abnormal vulva	% sterile	*n*
n2520/oz166	*n2520/n2520*	0	65	0	0	65
	n2520/oz166	14	116	16	0	100
	oz166/oz166	100	60	ND	ND	
n2520/nDf41	*n2520/nDf41*	1	83	0	1	82
n1924/oz166	*n1924/n1924*	0	48	2	0	48
	n1924/oz166	29	94	0	1	67
	oz166/oz166	85	48	100	100	7
n1925/oz166	*n1925/n1925*	0	46	0	0	46
	n1925/oz166	13	100	1	0	87
	oz166/oz166	100	49	ND	ND	
n2506/+ ♂ oz166/+ ⚥	*n2506/oz166*	15	74	100	0	21
n2506/+ ♂ nDf41/+ ⚥	*n2506/nDf41*	2	80	100	0	38
n2506/oz166	*n2506/n2506*	96	109	ND	ND	
	n2506/oz166	99	220	ND	ND	
	oz166/oz166	95	109	ND	ND	
n2506/nDf41	*n2506/n2506*	90	258	100	0	25
	n2506/nDf41	96	515	100	4	23
oz166/+ ♂ nDf41/+ ⚥	*oz166/nDf41*	6	120	100	100	48

Unless otherwise noted, columns are as defined in Table 1.

[a] The complete genotypes of parent hermaphrodites and F_1 progeny were as follows: *lin-45(n2520) unc-24/lin-45(oz166) dpy-20* parents generated F_1 progeny of genotype *lin-45(n2520) unc-24* (Unc nonDpy), *lin-45(n2520) unc-24/lin-45(oz166) dpy-20* (nonUnc nonDpy), and *lin-45(oz166) dpy-20* (larval lethal, occasionally surviving long enough to display the Dpy phenotype); *lin-45(n2520) unc-24/nDf41* parents generated F_1 progeny of genotype *lin-45(n2520) unc-24/nDf41* that were nonUnc. *nDf41* homozygotes display embryonic lethality; *lin-45(n1924) unc-24/lin-45(oz166) dpy-20* parents and *lin-45(1925) unc-24/lin-45(oz166) dpy-20* parents each generated F_1 progeny of three genotypes that were identified as described above; *lin-45(n2506) unc-24/dpy-20* males were crossed to *lin-45(oz166) dpy-20/bli-6 unc-24* hermaphrodites to generate *lin-45(n2506) unc-24/lin-45(oz166) dpy-20* hermaphrodite F_1 cross-progeny that were nonDpy, nonUnc, and did not segregate Bli Unc self-progeny. Nearly all self-progeny of these hermaphrodites displayed larval lethality, although 11 out of 428 survived to form an adult; *lin-45(n2506) unc-24/dpy-20* males were crossed to *nDf41/unc-5 dpy-20* hermaphrodites to generate *lin-45(n2506) unc-24/nDf41* hermaphrodite F_1 cross-progeny that were nonDpy, nonUnc, and did not segregate Dpy self-progeny. *nDf41* fails to complement *unc-5* and complements *unc-24* and *dpy-20*. Most self progeny of these hermaphrodites displayed larval lethality, and the genotype of surviving adults was inferred by the Unc/nonUnc phenotype; *lin-45(oz166)/unc-5 dpy-20* males were crossed to *nDf41/unc-5 dpy-20* hermaphrodites to generate *lin-45(oz166)/nDf41* hermaphrodite F_1 cross-progeny that were nonUnc nonDpy and did not segregate Unc Dpy self-progeny.

indicate that *n2520*, *n1924*, and *n1925* affect the function of *lin-45* during embryonic and/or larval development, since these mutations failed to complement the larval lethality caused by *oz166*.

On the basis of these findings, these alleles can be arranged in a series of increasing severity that is likely to correspond to an increasing loss of *lin-45* activity; the series is the same whether larval lethality, vulval formation, or sterility are considered. *n2520*, *n1924*, and *n1925* are weak alleles. These mutations do not cause highly penetrant defects in a wild-type genetic background. However, they affect vulval development, since they suppress the Muv defect caused by the *let-60(gf)* mutation, and they affect viability, since they fail to complement the larval lethality caused by *lin-45(oz166)*. There is no evidence that they affect the function of *lin-45* in the germline, since they complement the sterility caused by *lin-45(oz166)*. However, these mutations may affect the germline in a subtle way that was not detected by scoring progeny production. *n2018* and *n2506* are intermediate-strength alleles. These mutations cause partially penetrant larval lethality and vulval defects. Like the weak alleles, these mutations do not cause significant sterility, and *n2506* complemented the sterility caused by *lin-45(oz166)* (Table 2, line 11). The analysis of Han *et al.* (1993) indicates that *lin-45(sy96)* is also intermediate in strength. *n2510*, *oz166*, *oz178*, *oz201*, *dx19*, *dx84*, and *dx89* are strong alleles. These mutations caused completely penetrant sterile and Vul phenotypes and *oz166* caused completely penetrant lethality when the maternal contribution of *lin-45* was reduced.

The strong *lin-45* mutations are probably null alleles: Three types of evidence indicate that the strong mutations cause a very severe or complete loss of *lin-45* activity. First, these seven mutations all cause a very similar phenotype, and they are the strongest in the allelic

series. Second, the molecular analysis suggests that several of the mutant proteins have no activity; *dx19* and *n2510* encode truncated proteins that completely lack the kinase domain and *dx84* encodes a protein lacking a large region of the kinase domain. To rigorously test this hypothesis, we compared *oz166*, one of the strong alleles, to *nDf41*, a deficiency allele that deletes the *lin-45* locus as well as genes positioned right and left of *lin-45* (Table 2). Comparing *n2520/oz166* to *n2520/nDf41* (Table 2, lines 2 and 4), *n2506/oz166* to *n2506/nDf41* (Table 2, lines 11, 12, 14, and 17), and *oz166/oz166* to *oz166/nDf41* (Table 1, line 12, and Table 2, line 18) reveals that the defects caused by *nDf41* were similar to or less severe than the defects caused by *oz166*. These observations strongly support the hypothesis that *oz166* and the other six strong alleles are null mutations. However, it is possible that *oz166* retains some *lin-45* function that was not measured in these assays. The finding that *oz166* caused defects that were slightly more severe than those caused by *nDf41* might indicate that *oz166* causes some dominant negative effects or that *nDf41* deletes a gene(s) that affects the Ras pathway in addition to *lin-45*. *oz166* does not cause a strong dominant negative effect, since *oz166*/+ animals do not display noticeable defects (Table 1, line 13).

***lin-45* raf is essential for viability, fertility, and the induction of vulval cell fates:** Because the previously characterized *lin-45 (sy96)* allele is a partial loss-of-function mutation, the analysis of this allele did not demonstrate whether *lin-45* is essential for the processes that are affected in these mutants (Han *et al.* 1993). We used the probable null alleles of *lin-45* to address these questions. *lin-45* appears to be essential for viability, since 100% of *oz166* mutants derived from *n2520/oz166* or *n1925/oz166* hermaphrodites displayed larval lethality (Table 2, lines 3 and 10). *lin-45* is likely to promote viability by regulating the differentiation of the excretory duct cell (Yochem *et al.* 1997). *lin-45* appears to be essential for fertility, since 100% of homozygous mutants containing a strong mutation displayed sterility (Table 1). *lin-45* is likely to promote fertility by regulating germ cell progression through the pachytene stage (Church *et al.* 1995). One hundred percent of homozygous mutants containing a strong *lin-45* allele displayed an absence of vulval formation when viewed with a dissecting microscope. To further characterize vulval development, we used Nomarski optics to examine the fate of the vulval precursor cells in L4 stage hermaphrodites. In *oz166* mutants, P5.p, P6.p, and P7.p always generated two descendants that appeared to adopt the nonvulval 3° fate, resulting in a total of six descendants (Table 1, line 12). The same defect is caused by ablation of the anchor cell or severe loss-of-function mutations in *mek-2* and *mpk-1* (Horvitz and Sternberg 1991; Kornfeld *et al.* 1995a; Lackner and Kim 1998). These observations demonstrate that *lin-45* is essential for vulval precursor cells to adopt a vulval fate.

***lin-45* mutations exhibit complex interactions with a *let-60(gf)* mutation:** Six of the *lin-45* mutations were identified as suppressors of the Muv phenotype caused by a *let-60(gf)* mutation. *n1924*, *n1925*, *n2520*, *n2523*, and *n2506* can be maintained as homozygous strains and reduced the penetrance of the *let-60(gf)* Muv phenotype from 90 to 1% or less (Table 3). When the *lin-45(n2506)* mutation was separated from the *let-60(gf)* mutation and examined in a *let-60(+)* genetic background, several interesting features were apparent. Compared to the *lin-45(n2506) let-60(gf)* double mutants, the *lin-45(n2506)* single mutants displayed significantly more larval lethality (86 *vs.* 26%) and abnormal vulval development (93 *vs.* 4%) (Table 1, line 6, and Table 3, line 6). These observations suggest that the *let-60(gf)* mutation suppresses the larval lethal and vulvaless defects caused by *lin-45(n2506)* at the same time that *lin-45(n2506)* suppresses the multivulva defect caused by *let-60(gf)*. The increased activity of the mutant Ras and the decreased activity of the mutant Raf appear to be balanced such that the double mutant is more like wild type than either single mutant. It is noteworthy that the *lin-45(n2506)* mutation affects a residue in the Ras-binding domain.

lin-45(n2510) was identified as a dominant suppressor of the *let-60(gf)* Muv phenotype: 28% of *lin-45(n2510) let-60(gf)*/+ *let-60(gf)* animals displayed the Muv phenotype (Table 3, line 8). *lin-45(n2510) let-60(gf)* homozygous mutants displayed highly penetrant larval lethality (91%) and surviving adults were sterile and vulvaless (Table 3, line 7). Interestingly, when *lin-45(n2510)* was separated from *let-60(gf)*, the homozygous *lin-45(n2510)* mutants displayed a lower penetrance of 30% larval lethality (Table 1, line 9). These observations suggest that the *let-60(gf)* mutation enhances the larval lethality caused by *lin-45(n2510)*. To determine whether this result is typical of strong *lin-45* alleles, we generated a recombinant chromosome containing *lin-45(oz166)* and the *let-60(gf)* mutation. Whereas 68% of *lin-45(oz166)* animals displayed larval lethality (Table 1, line 12), 98% of *lin-45(oz166) let-60(gf)* animals displayed larval lethality (Table 3, line 9). Thus, the *let-60(gf)* mutation enhanced the larval lethality caused by two different strong *lin-45* mutations, suggesting this is a general phenomenon.

It is surprising that the *let-60(gf)* mutation suppressed the larval lethality caused by *lin-45(n2506)* and enhanced the larval lethality caused by *lin-45(n2510)* and *lin-45(oz166)*. One possible explanation is that the *let-60(gf)* mutation both positively and negatively affects the activity of the *let-60* gene. The positive effect is evident in combination with *lin-45(n2506)*, whereas the negative effect is evident in combination with *lin-45(n2510)* and *lin-45(oz166)*.

Comparison of strong loss-of-function mutations of *lin-45*, *mek-2*, and *mpk-1*: Strong loss-of-function or null mutations in the *mek-2* MAP kinase kinase and *mpk-1* ERK MAP kinase genes have been identified and charac-

TABLE 3

Interactions between *lin-45* mutations and a *let-60(gf)* mutation

Genotype of parent[a]	Genotype of F_1 progeny[a]	% larval lethal	*n*	% Muv[b]	% abnormal vulva	% sterile	*n*
let-60(gf)	*let-60(gf)*	13	189	78	ND	2	189
n1924 let-60(gf)	*n1924 let-60(gf)*	ND		0.3	ND	ND	650
n1925 let-60(gf)	*n1925 let-60(gf)*	ND		0.6	ND	ND	655
n2520 let-60(gf)	*n2520 let-60(gf)*	ND		0.5	ND	ND	604
n2523 let-60(gf)	*n2523 let-60(gf)*	ND		0.8	ND	ND	491
n2506 let-60(gf)	*n2506 let-60(gf)*	26	137	1	4	9	100
n2510 let-60(gf)/+ let-60(gf)	*n2510 let-60(gf)*	91	96	0	100	100	9
	n2510 let-60(gf)/+ let-60(gf)	ND		28	ND	ND	170
oz166 let-60(gf)/+ let-60(gf)	*oz166 let-60(gf)*	98	52	0	100	100	1
	oz166 let-60(gf)/+ let-60(gf)	ND		28	ND	ND	85

Unless otherwise noted, columns are as defined in Table 1.

[a] The complete genotypes of parent hermaphrodites and F_1 progeny were as follows: *let-60(n1046)*; *lin-45(n1924) let-60(n1046)*; *lin-45(n1925) let-60(n1046)*; *lin-45(n2520) let-60(n1046)*; *lin-45(n2523) let-60(n1046)*; *lin-45(n2506) let-60(n1046)*; and *lin-45(n2510) let-60(n1046)/let-60(n1046) dpy-20* parents generated nonDpy F_1 progeny of genotype *lin-45(n2510) let-60(n1046)* (lethal or sterile) and *lin-45(n2510) let-60(n1046)/let-60(n1046) dpy-20* (segregate Dpy self-progeny); *lin-45(oz166) let-60(n1046)/unc-24 let-60(n1046)* parents generated nonUnc F_1 progeny of genotype *lin-45(oz166) let-60(n1046)* (lethal or sterile) and *lin-45(oz166) let-60(n1046)/unc-24 let-60(n1046)* (segregate Unc self-progeny).

[b] For lines 2–5, we scored all the adult hermaphrodites on several petri dishes with a mixed-stage population for the Muv phenotype, one or more ventral protrusions displaced from the position of the vulva. For the other lines, adult hermaphrodites were descended from eggs placed on separate petri dishes.

terized (Church *et al.* 1995; Kornfeld *et al.* 1995a; Wu *et al.* 1995; Lackner and Kim 1998). Like the *lin-45(null)* mutations, *mek-2(null)* and *mpk-1(null)* mutations cause a partially penetrant larval lethal phenotype and a fully penetrant sterile and vulvaless phenotype in homozygous mutants derived from heterozygous hermaphrodites. Interactions between mutations that cause a Muv phenotype and loss-of-function mutations of *lin-45*, *mek-2*, and *mpk-1* have been analyzed previously (Han *et al.* 1993; Lackner *et al.* 1994; Wu and Han 1994; Wu *et al.* 1995; Kornfeld *et al.* 1995a; Lackner and Kim 1998; Tan *et al.* 1998). However, these studies were not performed with a *lin-45(null)* mutation. To directly compare the role of these three genes, we analyzed vulval development in double mutants containing a mutation that causes a Muv phenotype and a strong loss-of-function mutation in *lin-45*, *mek-2*, or *mpk-1*.

lin-15 is a complex locus that encodes one class A and one class B synthetic multivulva gene; *lin-15(n309)* strongly reduces the activity of both genes and causes a fully penetrant, highly expressive Muv phenotype (Clark *et al.* 1994). The Vul phenotype caused by strong *lin-45(lf)*, *mek-2(lf)*, and *mpk-1(lf)* mutations was fully epistatic to the *lin-15(lf)* Muv phenotype (Table 4, lines 1–7). Similarly, the Vul phenotype caused by these mutations was fully epistatic to the *let-60(gf)* Muv phenotype (Table 4, lines 8–14).

lin-1 encodes an ETS domain transcription factor that negatively regulates the 1° vulval cell fate, and loss-of-function mutations in *lin-1* cause a Muv phenotype (Beitel *et al.* 1995). The *lin-1(lf)* Muv phenotype was fully epistatic to the Vul phenotype caused by strong loss of function in *lin-45*, *mek-2*, and *mpk-1* (Table 4, lines 15–18). *lin-12* encodes a transmembrane receptor in the Notch family that promotes the 2° vulval cell fate, and it is likely that *lin-12* is normally activated in P5.p and P7.p by ligand produced by the 1° vulval cell, P6.p (Greenwald 1997). A gain-of-function mutation in *lin-12* causes a highly penetrant Muv phenotype, since all six Pn.p cells adopt the 2° vulval fate. The *lin-12* Muv phenotype was fully epistatic to the Vul phenotype caused by strong loss-of-function mutations in *lin-45*, *mek-2*, and *mpk-1* (Table 4, lines 28–31).

The predicted LIN-31 protein contains a winged helix domain and is likely to function as a transcription factor (Tan *et al.* 1998; Miller *et al.* 2000). A strong loss-of-function or null mutation of *lin-31* causes a partially penetrant Muv phenotype and a partially penetrant Vul phenotype, indicating that *lin-31* positively and negatively regulates vulval cell fates. The strong loss-of-function mutations *lin-45(oz166)* and *lin-45(oz201)* reduced the penetrance of the *lin-31* Muv phenotype from ~70% to 30 and 17%, respectively (Table 4, lines 19–23). By contrast, strong loss-of-function *mek-2* and *mpk-1* mutations did not reduce the penetrance of the *lin-31* Muv phenotype significantly (Table 4, lines 24–27). This is the first genetic background we identified in which the *lin-45(null)* mutations caused a significantly different phenotype than the *mek-2(null)* and *mpk-1(null)* mutations. These findings suggest that the expression of the *lin-31* Muv phenotype is partially dependent on *lin-45* activity but is not dependent on *mek-2* or *mpk-1* activity. Therefore, *lin-45* appears to have an activity that is not mediated by *mek-2* and *mpk-1*. Furthermore, this find-

TABLE 4

Interactions between *lin-45*, *mek-2*, and *mpk-1* and mutations that cause a Muv phenotype

Genotype[a]	% Muv	*n*
lin-15(lf)	100	624
lin-45(dx19)/+; lin-15(lf)	100	34
lin-45(dx19); lin-15(lf)	2	54
mek-2(n2516)/+; lin-15(lf)	100	123
mek-2(n2516); lin-15(lf)	0	213
mpk-1(ga117)/+; lin-15(lf)	100	38
mpk-1(ga117); lin-15(lf)	1	86
let-60(gf)	78	189
lin-45(n2510) let-60(gf)/+ let-60(gf)	28	170
lin-45(n2510) let-60(gf)	0	9
mek-2(n2516)/+; let-60(gf)	91	231
mek-2(n2516); let-60(gf)	0	156
mpk-1(ga117)/+; let-60(gf)	82	4335
mpk-1(ga117); let-60(gf)	0	882
lin-1(lf)	94	111
lin-1(lf) lin-45(oz166)	100	42
mek-2(n2516); lin-1(lf)	99	154
mpk-1(ga117); lin-1(lf)	97	29
lin-31(lf)	64	490
lin-45(oz166)/+; lin-31(lf)	78	369
lin-45(oz166); lin-31(lf)	30	80
lin-45(oz201)/+; lin-31(lf)	74	227
lin-45(oz201); lin-31(lf)	17	46
mek-2(n2678)/+; lin-31(lf)	77	94
mek-2(n2678); lin-31(lf)	72	60
mpk-1(ga117)/+; lin-31(lf)	68	164
mpk-1(ga117); lin-31(lf)	63	32
lin-12(gf)	98	284
lin-12(gf); lin-45(oz166)	100	51
mek-2(n2678); lin-12(gf)	100	399
mpk-1(ga117) lin-12(gf)	100	232

[a] The Muv mutations were *lin-15(n309lf)*X, *let-60(n1046gf)*IV, *lin-1(e1275 lf)*IV, *lin-31(n1053lf)*V, and *lin-12(n137gf)*III (see MATERIALS AND METHODS for a description of these alleles). *lin-45* double mutants with *lin-15*, *lin-31*, and *lin-12* were derived from *lin-45/unc-24 bli-6* parent hermaphrodites; sterile progeny were counted as *lin-45* homozygotes and fertile, non-Unc nonBli progeny were counted as *lin-45/+* heterozygotes. For *lin-45* double mutants with *let-60* and *lin-1*, which are on chromosome IV, the genotypes of the parent hermaphrodites were *lin-45(n2510) let-60(gf) +/+ let-60(gf) dpy-20* and *lin-1(lf) lin-45(oz166)/lin-1(lf) +*. *mek-2* double mutants were derived from *mek-2/sup-11 dpy-5* parent hermaphrodites; sterile progeny were counted as *mek-2* homozygotes, and fertile nonSup nonDpy progeny were counted as *mek-2/+* heterozygotes. *mpk-1* double mutants were derived from *mpk-1/unc-79 dpy-17* parent hermaphrodites; sterile progeny were counted as *mpk-1* homozygotes, and fertile, nonUnc nonDpy progeny were counted as *mpk-1/+* heterozygotes. For the *mpk-1* double mutant with *lin-12*, the genotype of the parent hermaphrodite was *mpk-1(ga117) lin-12(gf)/+ lin-12(gf)*. In general, fertility and vulval development were assessed by placing one L4 hermaphrodite on a petri dish and monitoring progeny production and vulval morphology using a dissecting microscope.

ing suggests that the position of *lin-31* in the signaling pathway may be complex, since it is not fully epistatic to *lin-45*.

DISCUSSION

***lin-45* is necessary for Ras-mediated signaling, and Raf, MEK, and ERK function in a predominantly linear signaling pathway:** We identified 13 mutations that reduce the activity of the *lin-45 raf* gene. These mutations can be arranged in an allelic series on the basis of the defects they caused in larval viability, fertility, and vulval development. Molecular and genetic analyses indicate that alleles that cause the most severe phenotype are likely to be null mutations. Previous analyses of the *lin-45(sy96)* mutation, which causes a partial loss of function, indicated that *lin-45* is important for larval viability, fertility, and vulval development (HAN *et al.* 1993). However, these studies did not resolve whether *lin-45* is essential for these processes, because the defects caused by *lin-45(sy96)* are partially penetrant. Our analysis of *lin-45(null)* alleles indicates that *lin-45* is essential for larval viability, fertility, and the induction of vulval cell fates, since *lin-45(null)* mutations cause completely penetrant larval lethality, sterility, and a vulvaless phenotype in which P5.p–P7.p are transformed to the 3° cell fate.

The MAP kinase kinase kinase protein family consists of multiple proteins, including Raf. Similarly, the MAP kinase kinase and MAP kinase families consist of multiple proteins, including MEK and ERK, respectively (FERRELL 1996). The finding that each of these protein families consists of multiple members has raised the possibility that more than one MAP kinase kinase kinase might phosphorylate a single MAP kinase kinase or that more than one MAP kinase kinase might phosphorylate a single MAP kinase (GARDNER *et al.* 1994). If this were the case during *C. elegans* development, then a null mutation in Raf might cause a less severe phenotype than a null mutation in MEK, since another MAP kinase kinase kinase could substitute for Raf and phosphorylate MEK. Similarly, if multiple MAP kinase kinases regulate ERK, then a null mutation in MEK might be expected to cause a less severe phenotype than a null mutation in ERK. To investigate these possibilities, we compared the phenotypes caused by null mutations in *lin-45 raf*, *mek-2* MEK, and *mpk-1* ERK MAP kinase. A mutation in each gene caused a completely penetrant vulvaless defect in which P5.p–P7.p adopt the 3° nonvulval cell fate (KORNFELD *et al.* 1995a; WU *et al.* 1995; LACKNER and KIM 1998). Mutations in each gene were completely epistatic to the Muv phenotype caused by a *lin-15(lf)* mutation and a *let-60(gf)* mutation, whereas the Muv phenotype caused by a *lin-1(lf)* and a *lin-12(gf)* mutation was completely epistatic to these Vul mutations. These results indicate that the loss of Raf activity is not less severe than the loss of MEK activity and that the loss of MEK activity is not less severe than the loss of ERK activity.

Thus, our results support the model that Raf is the only physiological activator of MEK and that MEK is the only physiological activator of ERK during these processes.

We did identify one genetic background in which the *lin-45(null)* mutation caused a more severe phenotype than the *mek-2(null)* or *mpk-1(null)* mutation. *lin-45(null)* mutations partially suppressed the Muv phenotype caused by a *lin-31(lf)* mutation, whereas *mek-2(null)* and *mpk-1(null)* mutations did not have this effect. These results demonstrate that *lin-45* activity is necessary for the full expression of the *lin-31* Muv phenotype, whereas *mek-2* and *mpk-1* activities do not appear to be necessary. However, it is possible that residual maternal *mek-2* and *mpk-1* activity account for this difference. One model that can explain these findings is that *lin-45* regulates a protein(s) in addition to MEK, and the regulation of this protein(s) is important for the *lin-31* Muv phenotype. According to this model, *lin-45* is a branchpoint in the signaling pathway. Taken together, our results indicate that *lin-45*, *mek-2*, and *mpk-1* function in a predominantly linear signaling pathway and raise the possibility that a small part of *lin-45* function is mediated by a protein(s) other than *mek-2*.

The Ras-binding domain, kinase domain, and 14-3-3-binding domain are necessary for Raf activity: It is important to identify and characterize the functional domains of Raf. Comparisons of Raf proteins that have diverged during evolution have identified conserved domains that are likely to be functionally significant. The mechanism of action of these domains can be investigated using biochemical experiments, whereas the functional significance of the domains can be investigated using genetic analysis. The genetic analysis of vertebrate Raf can yield results that are difficult to interpret, since mutant Raf is typically overexpressed in immortalized cultured cells and these cells also contain endogenous, wild-type Raf. By contrast, the *C. elegans* system described here overcomes many of these limitations. The *lin-45* mutations were present in both chromosomal copies, and thus mutant LIN-45 protein replaced wild-type LIN-45 in an otherwise wild-type animal. Furthermore, the use of random mutagenesis and screens for functional defects is a relatively unbiased way to identify functionally significant residues and domains. It is not completely unbiased, because chemical mutagens preferentially affect certain nucleotides and codons.

The interaction of Raf and Ras initiates Raf activation. This interaction has been characterized in a variety of binding assays (Vojtek *et al.* 1993; Zhang *et al.* 1993; Finney and Herrera 1995); the most detailed information about the structural basis for the Raf-Ras interaction comes from X-ray crystallography (Nassar *et al.* 1995). *lin-45(n2506 R118W)* affects a highly conserved arginine at the terminus of α-helix 1 that directly contacts Ras by hydrogen bonding to aspartic acid 38 and serine 39 of Ras (Figure 2). The finding that this mutation causes a significant reduction of Raf activity in worms supports the model that this residue is important for Raf binding to Ras. Remarkably, a mutation of Drosophila Raf that changes the homologous arginine 217 to leucine was discovered as an intermediate loss-of-function allele, *D-raf*^{C110} (Melnick *et al.* 1993). Introduction of this substitution of arginine 89 of vertebrate Raf reduces binding to Ras (Fabian *et al.* 1994; Block *et al.* 1996). Thus, the importance of this arginine for Raf binding to Ras has been highly conserved. The *lin-45(n2018 P92S)* mutation, which causes a moderate loss of function, and the *lin-45(n1925 R108W)* mutation, which causes a weak loss of function, affect residues in the Ras-binding domain that are not predicted to interact directly with Ras according to the crystal structure (Figure 2). Proline 92

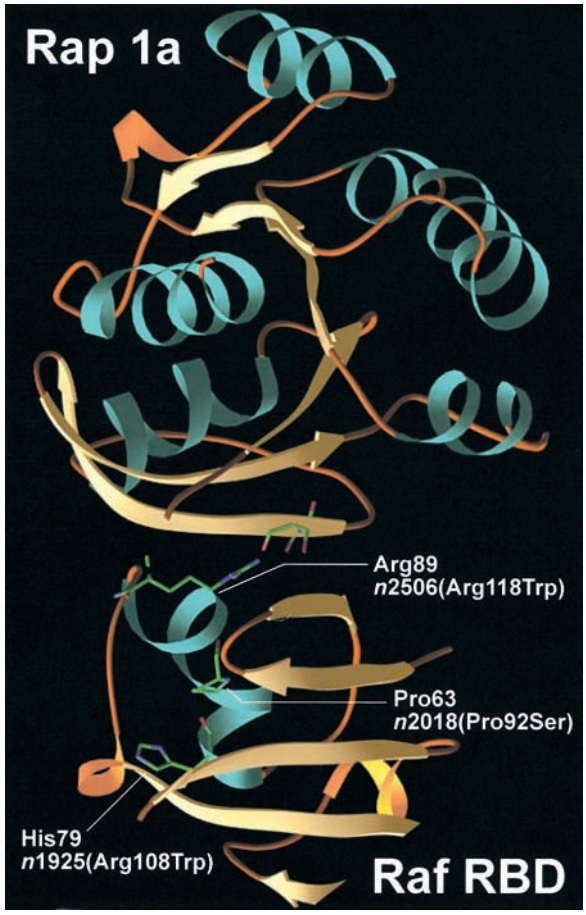

Figure 2.—Structure of the Ras-binding domain of Raf-1 complexed with Rap1A showing the position of the *lin-45* missense substitutions. Structure of the Ras-binding domain of human c-Raf1 (RBD, Ser51–Leu131) bound to truncated Rap1A complexed with GTP determined by X-ray crystallography (Nassar *et al.* 1995). Rap1A is a small GTP-binding protein with an effector region identical to Ras. β-sheets are yellow, α-helixes are blue, and other regions are orange. The side chains are shown for amino acids of human Raf-1 that are homologous to the residues of LIN-45 affected by missense mutations *n2018* (P92 LIN-45/P63 Raf-1), *n1925* (R108 LIN-45/H79 Raf-1), and *n2506* (R118 LIN-45/R89 Raf-1). The side chains of Rap1A amino acids Asp38 and Ser39 are shown, since these directly contact Arg 89 by hydrogen bonds.

is between β1-sheet and β2-sheet; both β-sheets contain residues predicted to directly contact Ras (Nassar *et al.* 1995). Arginine 108 is in α-helix 1 but positioned away from Ras. It is likely that the substitution of these residues disturbs the conformation of the Ras-binding domain and reduces the affinity of Raf and Ras.

The *lin-45(oz178 S645N)* mutation, which causes a strong loss of function, and the *lin-45(n1924 I726F)* mutation, which causes a weak loss of function, affect residues in the protein kinase domain. Serine 645 is a highly conserved residue, and our findings support the model that the substitution of this residue strongly reduces the kinase activity and kinase activity is essential for Raf function. Isoleucine 726 is moderately conserved—human Raf has valine at this position. Our findings support the model that this residue plays a secondary role in the function of the kinase domain. Mutations that affect highly conserved residues of the kinase domain of Drosophila Raf also cause a strong loss of function (Melnick *et al.* 1993).

14-3-3 proteins can bind to several phosphoserine motifs, including the RSXpSXP motif (Muslin *et al.* 1996). Raf contains two evolutionarily conserved RSXSXP motifs: an N-terminal motif in CR2 and a C-terminal motif following the kinase domain. Mutational analysis of vertebrate Raf indicates that the N-terminal 14-3-3 binding site negatively regulates Raf activity (Michaud *et al.* 1995; Rommel *et al.* 1996, 1997; Roy *et al.* 1997; McPherson *et al.* 1999). 14-3-3 binding to the C-terminal site has been suggested to be important for Raf function, since mutations in this motif reduce the function of Raf-1 expressed in cultured cells and the kinase activity of purified Raf-1 (Thorson *et al.* 1998; Tzivion *et al.* 1998). The *lin-45(n2520 S754F)* mutation changes the conserved 14-3-3 binding site from RSXSXP to RFXSXP and partially reduces the activity of *lin-45* in the worm. These findings indicate that this serine is important for 14-3-3 binding and/or for the phosphorylation of this motif by a protein kinase. Consistent with the possibility that this mutation reduces 14-3-3 binding, phosphorylated peptides with substitutions of alanine for this serine display reduced binding to 14-3-3 (Muslin *et al.* 1996). Our findings indicate that 14-3-3 binding to this position of Raf is necessary for full Raf activity and support the model that 14-3-3 binding to the C terminus positively regulates Raf.

Our findings have an additional implication for the role of 14-3-3 proteins in Ras-mediated signaling in *C. elegans*. *C. elegans* has multiple genes encoding 14-3-3 proteins (Wang and Shakes 1997). If 14-3-3 binding to Raf promotes Raf activity, then a mutant that lacks 14-3-3 protein is likely to have reduced Raf activity and reduced signal transduction. Mutations in *C. elegans* genes encoding 14-3-3 proteins have not been reported to display defects in Ras-mediated signaling. However, some genes have not been analyzed genetically. Genetic analysis in yeast and Drosophila has demonstrated that 14-3-3 proteins play a positive role in Ras-ERK signaling (Chang and Rubin 1997; Kockel *et al.* 1997; Li *et al.* 1997; Roberts *et al.* 1997). Our findings suggest that 14-3-3 proteins play a positive role in Ras-mediated signaling in *C. elegans* by promoting the activity of Raf.

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LITERATURE CITED

Alessi, D. R., Y. Saito, D. G. Campbell, P. Cohen, G. Sithanandam *et al.*, 1994 Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO J. **13:** 1610–1619.

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.

Block, C., R. Janknecht, C. Herrmann, N. Nassar and A. Wittinghofer, 1996 Quantitative structure-activity analysis correlating Ras/Raf interaction in vitro to Raf activation in vivo. Nat. Struct. Biol. **3:** 244–251.

Bonner, T. I., H. Oppermann, P. Seeburg, S. B. Kerby, M. A. Gunnell *et al.*, 1986 The complete coding sequence of the human raf oncogene and the corresponding structure of the *c-raf-1* gene. Nucleic Acids Res. **14:** 1009–1015.

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

Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani *et al.*, 1991 Oncogenes and signal transduction. Cell **64:** 281–302.

Chang, H. C., and G. M. Rubin, 1997 14–3-3ε positively regulates Ras-mediated signaling in *Drosophila*. Genes Dev. **11:** 1132–1139.

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.

Clark, S. G., M. J. Stern and H. R. Horvitz, 1992 *C. elegans* cell-signaling gene *sem-5* encodes a protein with SH2 and SH3 domains. Nature **356:** 340–344.

Clark, S. G., X. Lu and H. R. Horvitz, 1994 The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. Genetics **137:** 987–999.

Coulondre, C., and J. H. Miller, 1977 Genetic studies of the *lac* repressor. IV. Mutagenic specificity in the *LacI* gene of *Escherichia coli*. J. Mol. Biol. **117:** 577–606.

Denburg, A. F., K. McDonald, G. Moulder, R. Barstead, M. Dresser *et al.*, 1998 Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell **94:** 387–398.

Dickson, B., and E. Hafen, 1994 Genetics of signal transduction in invertebrates. Curr. Opin. Genet. Dev. **4:** 64–70.

Fabian, J. R., A. B. Vojtek, J. A. Cooper and D. K. Morrison, 1994 A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. USA **91:** 5982–5986.

Ferrell, J. E., 1996 MAP kinases in mitogenesis and development. Curr. Top. Dev. Biol. **33:** 1–60.

Finney, R., and D. Herrera, 1995 Ras-Raf complexes: analyses of complexes formed in vivo. Methods Enzymol. **255:** 310–323.

Gardner, A. M., R. R. Vaillancourt, C. A. Lange-Carter and G. L. Johnson, 1994 MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity. Mol. Biol. Cell **5:** 193–201.

Greenwald, I., 1997 Development of the vulva, pp. 519–541 in *C. elegans II*, edited by D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Greenwald, I., and G. Seydoux, 1990 Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. Nature **346:** 197–199.

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.

Heidecker, G., M. Huleihel, J. L. Cleveland, W. Kolch, T. W. Beck *et al.*, 1990 Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10:** 2503–2512.

Horvitz, H. R., and P. W. Sternberg, 1991 Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. Nature **351:** 535–541.

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.

Jakubowski, J., and K. Kornfeld, 1999 A local, high-density, single-nucleotide polymorphism map used to clone *Caenorhabditis elegans cdf-1*. Genetics **158:** 743–752.

Kockel, L., G. Vorbruggen, H. Jackle, M. Mlodzik and D. Bohmann, 1997 Requirement for Drosophila 14–3-3ζ in Raf-dependent photoreceptor development. Genes Dev. **11:** 1140–1147.

Kornfeld, K., 1997 Vulval development in *Caenorhabditis elegans*. Trends Genet. **13:** 55–61.

Kornfeld, K., K.-L. Guan and H. R. Horvitz, 1995a The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. Genes Dev. **9:** 756–768.

Kornfeld, K., D. B. Hom and H. R. Horvitz, 1995b The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans. Cell **83:** 903–913.

Lackner, M. R., and S. K. Kim, 1998 Genetic analysis of the *Caenorhabditis elegans* MAP kinase gene *mpk-1*. Genetics **150:** 103–117.

Lackner, M. R., K. Kornfeld, L. M. Miller, H. R. Horvitz and S. K. Kim, 1994 A MAP kinase homolog, *mpk-1*, is involved in *ras*-mediated induction of vulval cell fates in *Caenorhabditis elegans*. Genes Dev. **8:** 160–173.

Li, W., E. M. C. Skoulakis, R. L. Davis and N. Perrimon, 1997 The *Drosophila* 14–3-3 protein Leonardo enhances Torso signaling through D-Raf in a Ras1 dependent manner. Development **124:** 4163–4171.

Marais, R., Y. Light, H. F. Paterson and C. J. Marshall, 1995 Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. **14:** 3136–3145.

Marshall, C. J., 1994 MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. **4:** 82–89.

McPherson, R. A., A. Harding, S. Roy, A. Lane and J. F. Hancock, 1999 Interactions of c-Raf-1 with phosphatidylserine and 14–33. Oncogene **18:** 3862–3869.

Melnick, M. B., L. A. Perkins, M. Lee, L. Ambrosio and N. Perrimon, 1993 Developmental and molecular characterization of mutations in the *Drosophila-raf* serine/threonine protein kinase. Development **118:** 127–138.

Michaud, N. R., J. R. Fabian, K. D. Mathes and D. K. Morrsion, 1995 14–3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14–3-3 and Ras-independent manner. Mol. Cell. Biol. **15:** 3390–3397.

Miller, L. M., H. A. Hess, D. B. Doroquez and N. M. Andrews, 2000 Null mutations in the *lin-31* gene indicate two functions during *Caenorhabditis elegans* vulval development. Genetics **156:** 1595–1602.

Morrison, D. K., and R. E. Cutler, 1997 The complexity of Raf-1 regulation. Curr. Opin. Cell Biol. **9:** 174–179.

Muslin, A. J., J. W. Tanner, P. M. Allen and A. S. Shaw, 1996 Interaction of 14–3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell **84:** 889–897.

Nassar, N., G. Horn, C. Herrmann, A. Scherer, F. McCormick *et al.*, 1995 The 2.2 A crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. Nature **375:** 554–560.

Nishida, Y., M. Hata, T. Ayaki, H. Ryo, M. Yamagata *et al.*, 1988 Proliferation of both somatic and germ cells is affected in the *Drosophila* mutants of *raf* proto-oncogene. EMBO J. **7:** 775–781.

Riddle, D. L., T. Blumenthal, B. J. Meyer and J. R. Priess (Editors), 1997 *C. elegans II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Roberts, R. L., H.-U. Mosch and G. R. Fink, 1997 14–3-3 proteins are essential for RAS/MAPK cascade signaling during pseudohyphal development in S. cerevisiae. Cell **89:** 1055–1065.

Rommel, C., G. Radziwill, J. Lovric, J. Noeldeke, T. Heinicke *et al.*, 1996 Activated Ras displaces 14–3-3 protein from the amino terminus of c-Raf-1. Oncogene **12:** 609–619.

Rommel, C., G. Radziwill, K. Moelling and E. Hafen, 1997 Negative regulation of Raf activity by binding of 14–3-3 to the amino terminus of Raf in vivo. Mech. Dev. **64:** 95–104.

Roy, S., A. Lane, J. Yan, R. McPherson and J. F. Hancock, 1997 Activity of plasma membrane-recruited Raf-1 is regulated by Ras via the Raf zinc finger. J. Biol. Chem. **272:** 20139–20145.

Stanton, V. P., Jr., D. W. Nichols, A. P. Laudano and G. M. Cooper, 1989 Definition of the human raf amino-terminal regulatory region by deletion mutagenesis. Mol. Cell. Biol. **9:** 639–647.

Sternberg, P. W., and M. Han, 1998 Genetics of RAS signaling in *C. elegans*. Trends Genet. **14:** 466–472.

Stokoe, D., S. G. Macdonald, K. Cadwallader, M. Symons and J. F. Hancock, 1994 Activation of Raf as a result of recruitment to the plasma membrane. Science **264:** 1463–1467.

Tan, P. B., M. R. Lackner and S. K. Kim, 1998 MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. Cell **93:** 569–580.

Thorson, J. A., L. W. K. Yu, A. L. Hsu, N.-Y. Shih, P. R. Graves *et al.*, 1998 14–3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol. Cell. Biol. **18:** 5229–5238.

Treisman, R., 1996 Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. **8:** 205–215.

Tzivion, G., Z. Luo and J. Avruch, 1998 A dimeric 14–3-3 protein is an essential cofactor for Raf kinase activity. Nature **394:** 88–92.

Vojtek, A. B., S. M. Hollenberg and J. A. Cooper, 1993 Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:** 205–214.

Wang, W., and D. C. Shakes, 1997 Expression patterns and transcript processing of *ftt-1* and *ftt-2*, two *C. elegans* 14–3-3 homologues. J. Mol. Biol. **268:** 619–630.

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

Wu, Y., and M. Han, 1994 Suppression of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. Genes Dev. **8:** 147–159.

Wu, Y., M. Han and K.-L. Guan, 1995 MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes Dev. **9:** 742–755.

Yochem, J., M. Sundaram and M. Han, 1997 Ras is required for a limited number of cell fates and not for general proliferation in *Caenorhabditis elegans*. Mol. Cell. Biol. **17:** 2716–2722.

Zhang, X., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge *et al.*, 1993 Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature **364:** 308–365.

Zheng, C. F., and K.-L. Guan, 1994 Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO J. **13:** 1123–1131.

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