Caenorhabditis elegans CNK-1 promotes Raf activation but is not essential for Ras/Raf signaling

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Connector enhancer of Ksr (CNK) is a conserved multidomain protein essential for Ras signaling in *Drosophila melanogaster* and thought to be involved in Raf kinase activation. However, the precise role of CNK in Ras signaling is not known, and mammalian CNKs are proposed to have distinct functions. *Caenorhabditis elegans* has a single CNK homologue, *cnk-1*. Here, we describe the role of *cnk-1* in *C. elegans* Ras signaling and its requirements for LIN-45 Raf activation. We find that *cnk-1* positively regulates multiple Ras signaling events during development, but, unlike *Drosophila* CNK, *cnk-1* does not appear to be essential for signaling. *cnk-1* mutants appear to be normal but show cell-type-specific genetic interactions with mutations in two other Ras pathway scaffolds/adaptors *ksr-1* and *sur-8*. Genetic epistasis using various activated LIN-45 Raf transgenes shows that CNK-1 promotes LIN-45 Raf activation at a step between the dephosphorylation of inhibitory sites in the regulatory domain and activating phosphorylation in the kinase domain. Our data are consistent with a model in which CNK promotes Raf phosphorylation/activation through membrane localization, oligomerization, or association with an activating kinase.

scaffold | vulva | lin-45

Ras signaling through the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) kinase cascade is highly conserved and is required repeatedly during development. Aberrant signaling can lead to cancer, and mutations in Ras and B-Raf are commonly found in human tumors (1, 2). The Ras GTPase is a peripheral membrane protein that, when active (GTP bound), recruits the Raf kinase to the membrane, where they can physically interact, resulting in Raf activation (3, 4). In the absence of signal, Raf kinase is held in an inactive state in the cytoplasm. It is known that Raf requires membrane localization, Ras binding, and changes in phosphorylation and oligomerization to become active (3–5). However, it is not known how Ras activation leads to the recruitment of Raf or how Raf phosphorylation and activation occurs.

Genetic screens in *Drosophila* and *Caenorhabditis elegans* have identified a number of genes that act genetically between Ras and Raf and may play a role in Raf activation. *Drosophila* kinase suppressor of Ras (KSR) and *C. elegans ksr-1* and *ksr-2* encode Raf-related proteins that act as scaffolds for Raf, MEK, and ERK (6–9). KSR constitutively associates with MEK and appears to transiently associate with Raf and ERK upon signaling (10). KSR links Raf to its substrate MEK but could play an additional role in Raf activation (11–14). *C. elegans sur-6* encodes a B-regulatory subunit of the protein phosphatase 2A (PP2A) holoenzyme, thought to function with KSR (15–17), and *sur-8* encodes a leucine-rich repeat protein that binds Ras and may facilitate interactions between Ras and Raf (18–20). KSR is essential for Ras signaling in both *C. elegans* and *Drosophila*, whereas SUR-6 and SUR-8 are accessory proteins that facilitate, but are not absolutely essential for, signaling.

Drosophila connector enhancer of Ksr (CNK) acts genetically between Ras and Raf and is required for Raf activation in *Drosophila* S2 cells (14, 21). *cnk*-null mutants are cell-lethal, suggesting that CNK is essential for Ras signaling *in vivo* but precluding a detailed assessment of its requirements (21). Based on its multidomain structure, CNK is proposed to function as an adaptor or scaffold. CNK is membrane-localized, binds Raf, and may promote Raf membrane localization (14, 21). However, *Drosophila* CNK can promote signaling independently of its Raf interaction domain, and the Raf interaction domain may serve to inhibit Raf activation in the absence of signal (22). Thus, the mechanism by which CNK promotes Raf activity is unclear. Overexpression studies of mammalian CNK1 and CNK2 suggest that CNK may have a more widespread role in signal transduction, including roles in the Rho and Ral GTPase signaling pathways (23–28).

Here, we describe a genetic analysis of the single CNK orthologue in *C. elegans*. Our studies show that CNK-1 promotes Raf activation at a step before Raf-activation-loop phosphorylation. Surprisingly, CNK-1 is not absolutely needed for Ras signaling but functions as an accessory factor similar to SUR-6 or SUR-8.

Materials and Methods

Molecular Analysis of *cnk-1* and Isolation of the *cnk-1(sv39)* Deletion Allele. The *cnk-1* genomic structure was determined by sequencing cDNA clone yk1166a09 and RT-PCR products amplified from a cDNA library by using primers predicted from genomic DNA sequence. Our results differ from the WormBase (www.wormbase.org) prediction for R01H10.8 in several ways: (*i*) We find no evidence for the predicted exon between our exons 3 and 4, (*ii*) we identified an extra exon (exon 10), and (*iii*) we identified an in-frame ATG 15 nucleotide upstream of that predicted by WormBase (see GenBank Accession no. DQ104391). The *cnk-1(sv39)* allele was isolated by using PCR to screen a deletion library of mutagenized N2 worms, as described in ref. 16, by using PCR primers CRo110/CNK-1.FOR2 (5′-CCA AAC TAG CAT AAT GTT GT-3′) and CRo111/CNK-1.REV.INNRE (5′-CCC AAT CAT CTT CAT CAT CT-3′). The *cnk-1(ok836)* allele was obtained from the *C. elegans* Gene Knockout Consortium. *sv39* is a 1,983-bp deletion that removes three exons. However, the deleted region was detected by PCR and Southern blot of *sv39*, suggesting that part of the deleted region is present elsewhere in the genome (data not shown). An in-frame start codon is present downstream of the deletion breakpoint, at the beginning of exon 5. *ok836* is a 2,143-bp deletion. PCR using

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Abbreviations: CNK, connector enhancer of KSR; DRaf, *Drosophila* Raf; ERK, extracellular signal-regulated kinase; KSR, kinase suppressor of Ras; MEK, mitogen-activated protein kinase kinase; Muv, multivulva; RNAi, RNA interference; VPC, vulval precursor cell; Vul, vulvaless.

Data Deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ104391).

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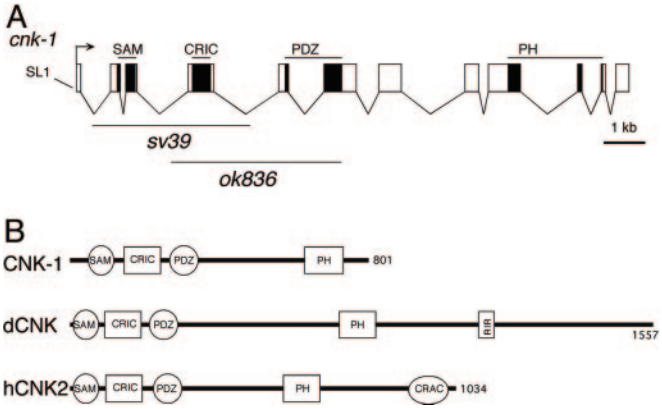

Fig. 1. Structure of the *cnk-1* gene and domains of the CNK-1 protein. (*A*) *cnk-1* genomic structure and location of deletion mutations. The exons are depicted as boxes and the introns as connecting lines. The black boxes depict regions encoding the sterile alpha motif (SAM), conserved region in Cnk (CRIC), PSD-90/Dlg/ZO-1 (PDZ), and Pleckstrin homology (PH) protein domains. The *sv39* and *ok836* deletions are represented as a line below the genomic structure (see *Materials and Methods*). (*B*) Schematic representation of CNK-1, Drosophila CNK, and human CNK2A proteins. RIR, Raf inhibitory region; CRAC, conserved region among chordates. CNK2A is the most closely related of the three mammalian CNK proteins to CNK-1 and also the only one so far implicated in Ras/Raf signaling (24, 26).

primers internal to the deletions was used to confirm that the deleted region was, indeed, absent in the *ok836* strain. RT-PCR analysis indicated that *ok836* produces an mRNA that preserves the ORF. Both *cnk-1* deletion alleles were outcrossed a minimum of six times against the wild-type N2 Bristol strain before analysis.

Genetics, Phenotype Analysis, and RNA Interference (RNAi). General methods for the handling and culturing of *C. elegans* were as described in ref. 29. Experiments were performed at 20°C, unless otherwise noted. Vulval, lethal, and 2 P11.p phenotypes were scored, as described in ref. 30. Multivulva (Muv) phenotypes were scored in L4 larvae by differential interference contrast optics. Animals with >3 vulval precursor cells (VPCs) induced were scored as Muv. RNAi was performed as described in ref. 31. Double-stranded RNA was prepared by *in vitro* transcription with T7 and T3 polymerases by using a PCR-derived *cnk-1* genomic template with primers oMS89 (5′-AAT TAA CCC TCA CTA AAG GGA TGG GAT TTC CGT CGA C-3′) and oMS90 (5′-GTA ATA CGA CTC ACT ATA GGG CCT AAC AAT TTG ACT-3′).

Generation of Transgenic Animals. Extrachromosomal array *csEx63* was used as a source of *hsp16–41-torso*^{4021}*-Draf* (*Drosophila* Raf) (G. Kao and M.S., unpublished data) and behaves identically to *csEx64* (16). *hsp16–41-lin-45ED* plasmid pCR33.1 was generated by cloning a PCR product amplified from plasmid lin-45ED (32) with primers CRo181 (5′-TTC ATG GCT AGC ACC ATG AGT CGG ATT AAT TTC AAA AAG TC-3′) and CRo182 (5′-TTC ATG CCA TGG CTA AAT GAG ACC ATA GAC ATT G-3′) into the NheI and NcoI sites of heat-shock vector pPD49.83. pCR33.1 was injected into N2 animals at 20 ng/μl with pTG_96 (*sur-5*::*gfp*) (33) at 30 ng/μl and pBluescript at 50 ng/μl to yield transgenic strain *csEx72*.

Results

C. elegans has a single CNK orthologue, *cnk-1* (Fig. 1). We determined the coding sequence of *cnk-1*, predicted gene *R01H10.8*, and found that it differs from the WormBase prediction (see *Materials and Methods*). *cnk-1* is predicted to encode

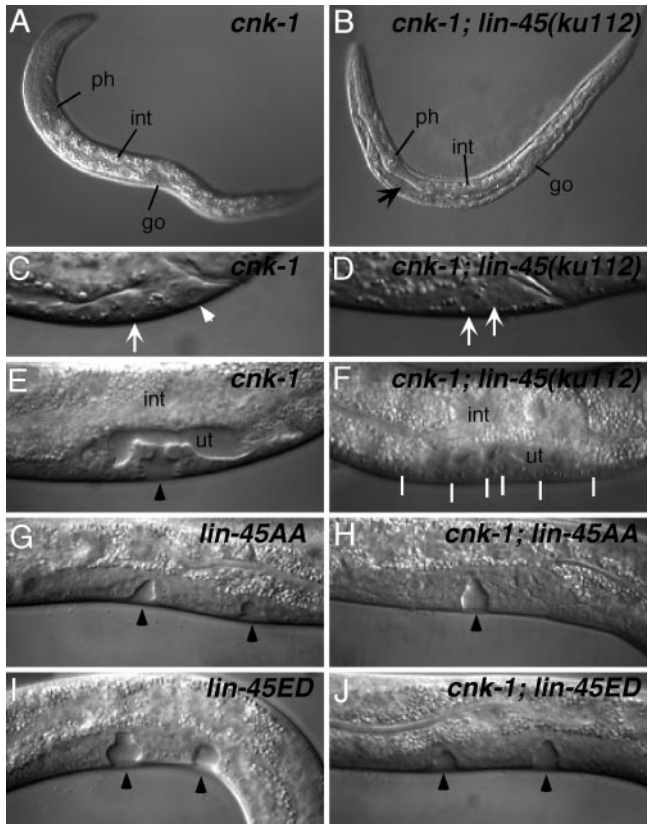

Fig. 2. *cnk-1* phenotypes in *lin-45 raf* mutant backgrounds. *cnk-1(sv39)* (*A*, *C*, and *E*) and *lin-45(ku112)* (30) mutants appear to be phenotypically normal, but *cnk-1(sv39); lin-45(ku112)* double mutants (*B*, *D*, and *F*) display strong Ras-like phenotypes. Differential interference contrast images of normal L1 larva (*A*) (ph, pharynx; int, intestine; go, gonad), rod-like lethal larva (*B*) (arrow marks fluid accumulation, which compresses the internal organs), normal P11.p (arrow), and P12.pa (arrowhead) cells (*C*), 2 P11.p (arrows) cells (*D*), normal L4 vulva (black arrowhead) (*E*), and vulvaless animal (*F*) (white lines mark uninduced cells). ut, uterus. *cnk-1(ok836)* suppresses *hs-lin-45AA* (*G* and *H*) but not *hs-lin-45ED* (*I* and *J*) Muv defects.

an 801-aa protein containing sterile alpha motif (SAM), a conserved region in Cnk (CRIC), PSD-90/Dlg/ZO-1 (PDZ), and Pleckstrin homology (PH) domains shared among other members of the CNK family of proteins. CNK-1 does not appear to have a Raf inhibitory region (RIR) domain specific to *Drosophila*, and we cannot detect direct physical interactions between CNK-1 and LIN-45 RAF in the yeast two-hybrid system (data not shown). CNK-1 also lacks a conserved-region-among-chordate (CRAC) domain specific to mammalian CNK proteins (22).

To determine whether *cnk-1* is required for Ras signaling, we screened a *C. elegans* deletion library by PCR and isolated a single deletion allele *sv39*. In addition, we obtained a second deletion allele *ok836* from the *C. elegans* Gene Knockout Consortium. Both deletions remove the most highly conserved regions of *cnk-1*, including the CRIC domain that has been shown to be essential for CNK function in *Drosophila* (21) (Fig. 1).

In *C. elegans*, Ras signaling is required for a number of developmental processes, including specification of vulval cell fates, the excretory duct cell, and the P12 cell (34). During vulval development, Ras signaling is required to induce three of six VPCs to undergo three rounds of division to produce the 22 cells that will form the vulva. Loss-of-function mutations in the Ras pathway result in a failure of the VPCs to be induced, causing a

Table 1. *cnk-1* displays synthetic phenotypes with *lin-45*, *ksr-1*, and *sur-8*

Genotype	Vul, %	Average no. of VPCs induced, (*n*)	Rod-like lethal, % (*n*)	2 P11.p, % (*n*)
cnk-1 (RNAi)	0	3.0 (100)	0 (> 1,000)	0 (100)
cnk-1 (sv39)	0	3.02 (45)*	0 (494)	2 (45)
cnk-1 (ok836)	0	3.00 (35)	0 (554)	0 (39)
cnk-1 (sv39)/nDf40†	0	3.00 (18)	0 (486)	0 (18)
lin-45 (ku112)	4	2.98 (24)	<1 (452)	0 (21)
cnk-1 (RNAi); lin-45 (ku112)	3	2.98 (61)	39 (1,413)‡	19 (62)
cnk-1 (sv39); lin-45 (ku112)	59‡	1.59 (58)	74 (712)‡	47 (59)‡
cnk-1 (sv39); lin-45 (ku112)† 25°C	25	2.56 (16)	ND	6 (16)
cnk-1 (sv39)/nDf40; lin-45 (ku112)† 25°C	21	2.47 (43)	ND	8 (36)
cnk-1 (ok836); lin-45 (ku112)	33§	2.49 (66)	89 (984)‡	24 (64)§
ksr-1 (n2526)	0	3.00 (26)	1 (180)	0 (24)
cnk-1 (RNAi); ksr-1 (n2526)	0	3.00 (71)	63 (1,146)‡	0 (71)
cnk-1 (sv39); ksr-1 (n2526)	0	3.00 (12)	95 (491)‡	6 (16)
cnk-1 (sv39)/+; ksr-1 (n2526)¶	ND	ND	3 (311)	ND
cnk-1 (ok836); ksr-1 (n2526)	0	3.00 (28)	98 (372)‡	4 (28)
sur-6(cs24)	2‖	2.97 (40)‖	0 (195)‖	0 (21)‖
sur-6(cs24); cnk-1 (sv39)	10	2.95 (20)	0 (110)	0 (20)
sur-8(ku167)	0	3.00 (25)	0 (328)	0 (24)
cnk-1 (RNAi); sur-8(ku167)	9	2.91 (69)	0 (942)	1 (69)
cnk-1 (sv39); sur-8(ku167)	28§	2.60 (25)	0 (343)	0 (25)
cnk-1 (ok836); sur-8(ku167)	14	2.93 (22)	3 (292)§	17 (23)§

Statistical analysis was performed by using Fisher's exact test (GraphPad). Statistical comparisons are made with the top genotype of each section. *lin-45 (ku112)* is a weak hypomorphic allele and is somewhat cold-sensitive (30), *ksr-1 (n2526)* is a putative null allele (6), *sur-8(ku167)* is a strong hypomorphic allele (18), and *sur-6(cs-24)* is a hypomorphic allele (17). *n*, number of animals scored. ND, no data.

*1 animal had a shifted vulva resulting in >3 VPCs being induced.

†*cnk-1 (sv39)* is linked to *unc-119(e2498)* and deficiency *nDf40* is linked to *dpy-18(e364)*; described in ref. 48 and www.wormbase.org. *lin-45 (ku112)* is linked to *dpy-20(e1282)*. Experiments with *nDf40* in the *lin-45 (ku112)* background (and non-Df sibling controls) were performed at 25°C as indicated.

‡$P < 0.001$.

§$P < 0.05$.

¶Progeny from *cnk-1 (sv39); ksr-1(n2526)* hermaphrodites mated with *cnk-1(+): ksr-1 (n2526)* males.

‖Data from refs. 17 and 29.

Table 2. Epistasis with Muv strains

Genotype	Muv, %	Average no. of VPCs induced, (*n*)
let-60(n1046gf)	56	3.48 (27)
cnk-1 (sv39); let-60(n1046gf)	0*	3.00 (33)
cnk-1 (ok836)/hT2; let-60(n1046gf)	67	3.56 (24)
cnk-1 (ok836); let-60(n1046gf)	16*	3.11 (32)
hs-torso^{4021}-Draf	13	3.13 (106)
cnk-1 (sv39); hs-torso^{4021}-Draf	31†	3.38 (45)
cnk-1 (ok836); hs-torso^{4021}-Draf	21	3.19 (39)
gaIs36	87	5.02 (46)
cnk-1 (sv39); gaIs36	95	5.10 (19)
cnk-1 (ok836); gaIs36	61†	4.40 (36)
lin-45 (sy96); gaIs36	75	3.40 (12)
lin-1 (n304)	100	5.53 (17)
cnk-1 (ok836); lin-1 (n304)	100	5.78 (16)

Statistical analysis is the same as that described in Table 1. For epistasis with *cnk-1(sv39)*, *csEx63 (hsp16-41-torso4021-Draf)* larvae were heat-shocked at 38.5°C for 45 min, 48–50 h after egg-lay. For epistasis with *cnk-1(ok836)*, *csEx63* larvae were heat-shocked 30–34 h after plating synchronized L1 larvae. The integrated transgene *gaIs36* (*E1F-D-mek*, *hs-mpk-1)* was linked to *him-5(e1490)* (38, 48). *gaIs36*-bearing animals were shifted from 20°C to 25°C, and progeny were scored for the Muv phenotype. *gaIs36* may be sensitive to mutations upstream in the pathway because it is mildly suppressed by *cnk-1(ok836)* and *lin-45(sy96)*. *let-60(n1046)* is a gain-of-function allele (36, 37), *line-l(n304)* is null allele (39), *line-45(sy96)* is a strong hypomorphic allele (49), and *hT2[qIs48]* (50) balances the *cnk-1* locus. *n*, number of animals scored

*$P < 0.001$.

†$P < 0.05$.

vulvaless (Vul) phenotype. Ras signaling is required in the excretory duct cell for viability (35). Loss-of-function mutations in the Ras pathway cause a loss of the excretory duct cell fate, resulting in animals filling with fluid and dying in the first larval stage with a distinct "rod-like" appearance. Ras signaling is required for the P12 blast cell fate. Loss-of-function mutations in the Ras pathway cause P12 to adopt a fate similar to its neighbor P11, resulting in a loss of the P12.pa cell and duplication of P11.p. Animals homozygous for either *cnk-1* deletion allele appear to be essentially wild-type and display little or no discernible Ras phenotypes (Fig. 2 *A*, *C*, and *E* and Table 1). We believe that both *cnk-1* deletions are strong loss-of-function alleles. Although they could potentially produce partial protein products (see *Materials and Methods*), each would lack multiple conserved domains (Fig. 1). Furthermore, *cnk-1(RNAi)* is similar to, and no more severe than, either deletion allele, and we find no phenotype enhancement when *sv39* is placed in trans to a deficiency that removes *cnk-1*, and *cnk-1* is a recessive modifier of other Ras pathway mutants (Table 1 and see below). Thus, *cnk-1* does not appear to be essential for Ras signaling in *C. elegans*.

Several previously described positive regulators of *C. elegans* Ras signaling, such as *ksr-1*, *sur-6*, and *sur-8* have little or no Ras mutant phenotypes alone, but display strong Ras phenotypes in a genetically sensitized background (6, 7, 17, 18). For example, some hypomorphic alleles of *lin-45 raf* do not display overt Ras phenotypes but are strongly enhanced by the removal of *ksr-1* or *sur-6* (7, 17, 30). We find that both *cnk-1* alleles strongly synergize with the hypomorphic allele *lin-45(ku112)*. *cnk-1; lin-45(ku112*) animals display strong Ras phenotypes, including rod-like larval lethality, Vul, and 2 P11.p cell phenotypes (Fig. 2 *B*, *D*, and *F* and Table 1). Therefore, *cnk-1* is a positive regulator of Ras signaling similar to *ksr-1*, *sur-6*, and *sur-8*.

We tested for genetic interactions between *cnk-1* and *sur-6* PP2A-B and the scaffolds/adaptors *ksr-1*, *ksr-2*, and *sur-8*. *cnk-1* deletions show strong synergistic phenotypes with both *ksr-1* and *sur-8* but not *ksr-2* or *sur-6* (Table 1 and data not shown). Interestingly, the *cnk-1; ksr-1* doubles have a very highly penetrant rod-like larval lethal phenotype, but escapers have normal P12 and vulval fates. In contrast, *cnk-1; sur-8* animals display mild Vul and 2 P11.p phenotypes but little or no rod-like lethality. Thus there appear to be different requirements for these scaffolds/adaptors in different tissues.

DEVELOPMENTAL BIOLOGY

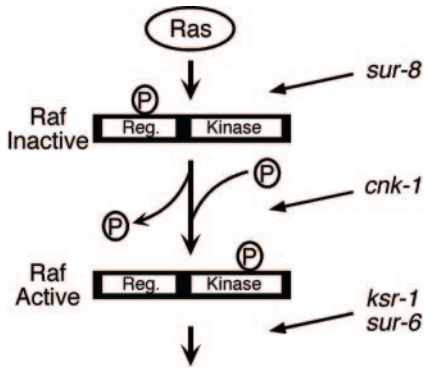

Fig. 3. Genetic epistasis data indicate that *cnk-1* promotes Ras signaling at a step of Raf activation after dephosphorylation of inhibitory sites in the regulatory domain (Reg.) and before activating phosphorylation in the kinase domain. This placement is distinct from *sur-8*, which is required upstream of the dephosphorylation event (45) and *ksr-1* and *sur-6*, which are required downstream of activating phosphorylation in the kinase domain. *ksr-1* and *sur-6* could also have additional, upstream requirements. Circled P, phosphate group.

Table 3. Epistasis with *lin-45* gain-of-function strains

Genotype	Muv, % (*n*)	Average no. of VPCs induced, (*n*)
hs-lin-45AA	91 (32)	4.75 (32)
cnk-1 (ok836); hs-lin-45AA	38 (34)*	3.40 (34)
hs-lin-45AA	50 (26)	3.81 (26)
cnk-1 (sv39); hs-lin-45AA	0 (30)*	3.00 (30)
hs-lin-45AA	76 (45)	4.68 (25)
let-23 (sy1); hs-lin-45AA	83 (47)	4.08 (25)
let-23 (sy1)	0 (27)†	0.80 (27)
dpy-20; hs-lin-45AA	88 (17)	4.62 (17)
let-60(sy94)/dpy-20; hs-lin-45AA	95 (19)	5.42 (19)
let-60(sy94)/dpy-20	0 (14)†	0.04 (14)
hs-lin-45ED	37 (30)	3.37 (30)
cnk-1 (ok836); hs-lin-45ED	48 (29)	3.84 (29)
sur-6(cs24); hs-lin-45ED	0 (28)*	3.00 (28)
ksr-1 (n2526); hs-lin-45ED	3 (29)‡	3.02 (29)

Statictical analysis is the same as that described in Table 1. Extrachromosomal array *csEx52* was used as a source of *hsp16-41-lin-45AA* (16). For epistasis with *cnk-1(sv39)*, *csEx52* larvae were heat-shocked at 37°C for 45 min 40.5–43.5 h after egg-lay. For others, *csEx52* larvae were heat-shocked at 39°C for 45 min 28 h (30 with *let-23*) after plating synchronized L1 larvae. *csEx72 (hsp16-41-lin-45ED)* larvae were heat-shocked at 38.5°C for 45 min 31 h after plating synchronized L1 larvae. *let-23(syl)* is a strong hypomorphic allele, *let-60(sy94)* is a dominant negative allele, and *dpy-20(e1282)* was used to balance *let-60* (48, 51). *n*, number of animals scored.

*$P < 0.001$.

†Animals are Vul (see text).

‡$P < 0.05$.

During vulval development, only three of six VPCs are induced to make vulval tissue, but when Ras signaling is inappropriately activated in the VPCs, >3 VPCs can be induced, resulting in a Muv phenotype (34). To determine where in the Ras pathway *cnk-1* functions, we performed genetic epistasis, testing the ability of the *cnk-1* deletions to suppress the Muv phenotype of different activated components of the Ras pathway. A gain-of-function allele of *let-60 ras* analogous to the oncogenic form of human Ras G13E induces a Muv phenotype (36, 37). We find that *cnk-1* significantly suppresses the Muv phenotype of *let-60 ras* gain-of-function, suggesting that *cnk-1* is required downstream of *let-60 ras* (Table 2). To determine where *cnk-1* acts with respect to Raf, we tested the ability of *cnk-1* to suppress the Muv phenotype caused by an activated DRaf chimera, Torso^{4021}-DRaf. A transgene expressing activated *torso^{4021}-Draf* under the control of the heat-shock promoter *hsp16–41* induces a Muv phenotype (18). *cnk-1* fails to suppress the Muv phenotype of *hs-torso^{4021}-Draf*, consistent with *cnk-1* acting upstream or in parallel to Raf (Table 2). An integrated transgene, *gaIs36*, expressing both an activated version of *Drosophila* MEK and *C. elegans mpk-1* ERK induces a potent Muv phenotype when grown at 25° (38). *cnk-1* fails to strongly suppress the Muv phenotype of *gaIs36* (Table 2). *lin-1* encodes an ETS domain protein that acts downstream of the pathway to negatively regulate vulval induction (39). We find that neither *cnk-1* allele can suppress the Muv phenotype of a *lin-1* loss-of-function mutant (Table 2). These data are consistent with CNK epistasis in *Drosophila* and place *cnk-1* genetically between Ras and Raf.

Raf kinase activation is a complex multistep process requiring relief of autoinhibition by the Raf N terminus and changes in phosphorylation, membrane localization, Ras binding, and oligomerization (3–5). The Torso^{4021}-DRaf chimera typically used for epistasis experiments in both *Drosophila* and *C. elegans* likely bypasses most of these steps (40, 41). This chimera consists of the extracellular and membrane-spanning domains of a constitutively dimerizing Torso receptor tyrosine kinase fused to a truncated DRaf missing the N-terminal Ras binding domain. The remaining C-terminal portion of Draf (containing the kinase domain) may escape autoinhibition and is predicted to be constitutively membrane-localized and oligomerized. Torso^{4021}-Draf can signal independently of the SUR-6 PP2A subunit and the SUR-8, KSR-1, and CNK-1 scaffolds (8, 17, 18, 21).

To better understand the proposed role of CNK in Raf activation, we used two more specific forms of activated *C. elegans* LIN-45 Raf. The first form, LIN-45(AA), lacks inhibitory phosphorylation sites in the N-terminal regulatory domain (32) (Fig. 3). The analogous residues in mammalian Raf proteins serve as docking sites for the 14-3-3 chaperone, which inhibits translocation of Raf to the membrane (42). Dephosphorylation of these inhibitory sites by PP2A is thought to be an early step in Raf activation (43). The second form of activated LIN-45 Raf, LIN-45(ED), contains negatively charged residues that mimic activating phosphorylation within the activation loop of the kinase domain (32) (Fig. 3). Phosphorylation of these activating sites by an unknown, membrane-localized kinase is thought to be a late step in Raf activation (43). Both LIN-45(AA) and LIN-45(ED) mutants can induce a Muv phenotype, when expressed as a transgene (32). We tested whether *cnk-1* mutants could suppress the Muv phenotype of these differentially activated LIN-45 Raf constructs.

Whereas *cnk-1* deletions were unable to suppress the Muv phenotype of *hs-torso^{4021}-Draf*, both deletions readily suppress the Muv phenotype of *hs-lin-45AA* (Fig. 2 *G* and *H* and Table 3). This finding suggests that either CNK-1 is required downstream of LIN-45 Raf AA or, alternatively, that LIN-45 AA activity depends on upstream signaling components. We do not believe that LIN-45 AA depends on upstream signal, because a strong mutation in the *let-23* receptor and a dominant-negative allele of *let-60 ras* (which are 85% and 100% Vul, respectively) fail to suppress the Muv phenotype of *hs-lin-45AA* (Table 3). Because LIN-45AA still requires CNK-1 to elicit a Muv phenotype, it is unlikely that CNK-1 plays a role in dephosphorylation of the Raf regulatory domain.

LIN-45 ED replaces activating phosphorylation sites Thr 626 and Thr 629 with glutamic acid and aspartic acid, respectively, to mimic constitutive phosphorylation. LIN-45 ED likely functions analogously to the most common oncogenic B-Raf mutation V599E, which would correspond to Val 627 in LIN-45 Raf (2, 44). We find that the *cnk-1* deletions cannot suppress the Muv phenotype of *hs-lin-45ED* (Fig. 2 *I* and *J* and Table 3). Thus,

CNK-1 is not required after activating phosphorylation in the kinase domain. Together, these data suggest that CNK-1 promotes the activation of LIN-45 Raf after dephosphorylation of the inhibitory sites in the regulatory domain and before activating phosphorylation in the kinase domain (Fig. 3). For example, CNK-1 could promote Raf membrane translocation, oligomerization, or association with the activating kinase.

LIN-45 AA has been used for epistasis analysis with *ksr-1*, *sur-6*, and *sur-8* (16, 45). Like *cnk-1*, *ksr-1* and *sur-6* mutations strongly suppress the Muv phenotype of *hs-lin-45AA*; however, a *sur-8* mutant is unable to suppress LIN-45 AA. Thus, *cnk-1*, *ksr-1*, and *sur-6* function at a distinct step of the pathway from *sur-8*. We tested whether *ksr-1* and *sur-6* can suppress the Muv phenotype of LIN-45 ED. Whereas *cnk-1* mutations cannot suppress the Muv phenotype of LIN-45 ED, both *ksr-1* and *sur-6* can suppress LIN-45 ED (Table 3). These data are consistent with the model in which KSR-1 and SUR-6 promote the ability of active Raf to access its substrate MEK but cannot exclude the possibility that these gene products have additional, earlier functions. Overall, our data suggest that CNK-1 and SUR-8 promote distinct steps of Raf activation and are not required for a later, KSR-1- and SUR-6-dependent step of signaling (Fig. 3).

Discussion

We have shown that *C. elegans cnk-1* promotes Ras signaling in several different tissues during development. However, *C. elegans cnk-1* does not appear to be an essential component of the Ras pathway. Our findings differ from those in *Drosophila*, where *cnk* appears to be essential for Ras signaling (14, 21). Because there is only one CNK gene in *C. elegans*, this difference may be due to redundancy with a nonhomologous protein or reflect a difference in how Raf is activated in *C. elegans* versus *Drosophila*. Alternatively, the lethality of *cnk*-null cells may be masking significant nonrequirements for *cnk* in *Drosophila*. Knockouts of the three mammalian CNK genes have not yet been described and whether their requirements will resemble those of *Drosophila* or *C. elegans* remains to be determined.

In *Drosophila*, CNK has both positive and negative regulatory roles in Ras signaling (22). Our genetic studies indicate that the primary role of *C. elegans* CNK-1 is to positively regulate Ras signaling. However, a potential negative role of CNK-1 is suggested by the observation that *cnk-1* mutations somewhat enhance the Torso^{4021}-DRaf Muv phenotype (Table 2). If CNK-1 were to play both positive and negative roles in Ras signaling, that dual function might help explain the mild phenotype caused by *cnk-1* mutations.

We find no evidence to suggest that *C. elegans cnk-1* functions outside of Ras signaling. In particular, we do not observe embryonic-lethality or cell-migration defects indicative of defects in *rho-1* signaling (46, 47). It is possible that alternative roles for *cnk-1* may be detectable only in the right genetically sensitized background. Notably, however, both *Drosophila* and *C. elegans* CNK proteins are most similar to mammalian CNK2, which has been found to affect Ras signaling (24, 26) and less similar to mammalian CNK1, which has been found to affect Rho signaling (25, 27, 28). It is possible that the multiple mammalian CNK proteins have evolved to serve different GTPase signaling pathways.

Interestingly, *cnk-1* shows cell-type-specific genetic interactions with *ksr-1* and *sur-8*. These genetic interactions may reflect differences in how signals are transmitted in different cell types and suggest that contributions of different scaffolds and adaptors might be one mechanism by which signaling specificity is achieved. The *cnk-1* epistasis analysis with LIN-45 Raf AA and LIN-45 Raf ED shows that the role of *cnk-1* is distinct from that of *ksr-1*, *sur-6*, and *sur-8* and further narrows down the possible mechanisms by which CNK functions to regulate Raf activation. Our data argue against a role for CNK in Raf regulatory domain dephosphorylation but are consistent with models in which CNK promotes Raf membrane localization, oligomerization, or association with an activating kinase.

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1. Bos, J. L. (1989) *Cancer Res.* **49,** 4682–4689.
2. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., *et al.* (2002) *Nature* **417,** 949–954.
3. Chong, H., Vikis, H. G. & Guan, K. L. (2003) *Cell. Signalling* **15,** 463–469.
4. Dhillon, A. S. & Kolch, W. (2002) *Arch. Biochem. Biophys.* **404,** 3–9.
5. Morrison, D. K. & Cutler, R. E. (1997) *Curr. Opin. Cell Biol.* **9,** 174–179.
6. Kornfeld, K., Hom, D. B. & Horvitz, H. R. (1995) *Cell* **83,** 903–913.
7. Sundaram, M. & Han, M. (1995) *Cell* **83,** 889–901.
8. Therrien, M., Chang, H. C., Solomon, N. M., Karim, F. D., Wassarman, D. A. & Rubin, G. M. (1995) *Cell* **83,** 879–888.
9. Ohmachi, M., Rocheleau, C. E., Church, D., Lambie, E., Schedl, T. & Sundaram, M. V. (2002) *Curr. Biol.* **12,** 427–433.
10. Morrison, D. K. (2001) *J. Cell Sci.* **114,** 1609–1612.
11. Michaud, N. R., Therrien, M., Cacace, A., Edsall, L. C., Spiegel, S., Rubin, G. M. & Morrison, D. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12792–12796.
12. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X. H., Basu, S., McGinley, M., Chan-Hui, P. Y., Lichenstein, H. & Kolesnick, R. (1997) *Cell* **89,** 63–72.
13. Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. (2002) *Genes Dev.* **16,** 427–438.
14. Anselmo, A. N., Bumeister, R., Thomas, J. M. & White, M. A. (2002) *J. Biol. Chem.* **277,** 5940–5943.
15. Ory, S., Zhou, M., Conrads, T. P., Veenstra, T. D. & Morrison, D. K. (2003) *Curr. Biol.* **13,** 1356–1364.
16. Kao, G., Tuck, S., Baillie, D. & Sundaram, M. V. (2004) *Development (Cambridge, U.K.)* **131,** 755–765.
17. Sieburth, D. S., Sundaram, M., Howard, R. M. & Han, M. (1999) *Genes Dev.* **13,** 2562–2569.
18. Sieburth, D. S., Sun, Q. & Han, M. (1998) *Cell* **94,** 119–130.
19. Li, W., Han, M. & Guan, K. L. (2000) *Genes Dev.* **14,** 895–900.
20. Selfors, L. M., Schutzman, J. L., Borland, C. Z. & Stern, M. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6903–6908.
21. Therrien, M., Wong, A. M. & Rubin, G. M. (1998) *Cell* **95,** 343–353.
22. Douziech, M., Roy, F., Laberge, G., Lefrancois, M., Armengod, A. V. & Therrien, M. (2003) *EMBO J.* **22,** 5068–5078.
23. Rabizadeh, S., Xavier, R. J., Ishiguro, K., Bernabeortiz, J., Lopez-Ilasaca, M., Khokhlatchev, A., Mollahan, P., Pfeifer, G. P., Avruch, J. & Seed, B. (2004) *J. Biol. Chem.* **279,** 29247–29254.
24. Bumeister, R., Rosse, C., Anselmo, A., Camonis, J. & White, M. A. (2004) *Curr. Biol.* **14,** 439–445.
25. Lopez-Ilasaca, M. A., Bernabe-Ortiz, J. C., Na, S. Y., Dzau, V. J. & Xavier, R. J. (2005) *FEBS Lett.* **579,** 648–654.
26. Lanigan, T. M., Liu, A., Huang, Y. Z., Mei, L., Margolis, B. & Guan, K. L. (2003) *FASEB J.* **17,** 2048–2060.
27. Jaffe, A. B., Aspenstrom, P. & Hall, A. (2004) *Mol. Cell. Biol.* **24,** 1736–1746.
28. Jaffe, A. B., Hall, A. & Schmidt, A. (2005) *Curr. Biol.* **15,** 405–412.
29. Brenner, S. (1974) *Genetics* **77,** 71–94.
30. Rocheleau, C. E., Howard, R. M., Goldman, A. P., Volk, M. L., Girard, L. J. & Sundaram, M. V. (2002) *Genetics* **161,** 121–131.
31. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) *Nature* **391,** 806–811.
32. Chong, H., Lee, J. & Guan, K. L. (2001) *EMBO J.* **20,** 3716–3727.
33. Yochem, J., Gu, T. & Han, M. (1998) *Genetics* **149,** 1323–1334.
34. Sternberg, P. W. & Han, M. (1998) *Trends Genet.* **14,** 466–472.
35. Yochem, J., Sundaram, M. & Han, M. (1997) *Mol. Cell. Biol.* **17,** 2716–2722.
36. Beitel, G. J., Clark, S. G. & Horvitz, H. R. (1990) *Nature* **348,** 503–509.
37. Han, M. & Sternberg, P. W. (1990) *Cell* **63,** 921–931.
38. Lackner, M. R. & Kim, S. K. (1998) *Genetics* **150,** 103–117.

39. Beitel, G. J., Tuck, S., Greenwald, I. & Horvitz, H. R. (1995) *Genes Dev.* **9,** 3149–3162.
40. Dickson, B., Sprenger, F., Morrison, D. & Hafen, E. (1992) *Nature* **360,** 600–603.
41. Baek, K. H., Fabian, J. R., Sprenger, F., Morrison, D. K. & Ambrosio, L. (1996) *Dev. Biol.* **175,** 191–204.
42. Jaumot, M. & Hancock, J. F. (2001) *Oncogene* **20,** 3949–3958.
43. Chong, H. & Guan, K. L. (2003) *J. Biol. Chem.* **278,** 36269–36276.
44. Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D. & Marais, R. (2004) *Cell* **116,** 855–867.
45. Yoder, J. H., Chong, H., Guan, K. L. & Han, M. (2003) *EMBO J.* **23,** 111–119.
46. Spencer, A. G., Orita, S., Malone, C. J. & Han, M. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 13132–13137.
47. Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A. & Glotzer, M. (2000) *J. Cell. Biol.* **149,** 1391–1404.
48. Riddle, D. L., Blumenthal, T., Meyer, B. J. & Priess, J. R. (1997) *C. elegans II* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY), pp. 902–1047.
49. Han, M., Golden, A., Han, Y. & Sternberg, P. W. (1993) *Nature* **363,** 133–140.
50. Wang, S. & Kimble, J. (2001) *EMBO J.* **20,** 1363–1372.
51. Han, M. & Sternberg, P. W. (1991) *Genes Dev.* **5,** 2188–2198.