

Modulation of KSR activity in *Caenorhabditis elegans* by Zn ions, PAR-1 kinase and PP2A phosphatase

John H Yoder^{1,3}, Huiira Chong², Kun-liang Guan² and Min Han^{1,*}

¹Department of Molecular, Cellular and Developmental Biology, Howard Hughes Medical Institution, University of Colorado, Boulder, CO, USA and ²Department of Biological Chemistry and The Institute of Gerontology, University of Michigan, Ann Arbor, MI, USA

Vulval differentiation in *Caenorhabditis elegans* is controlled by a conserved signal transduction pathway mediated by Ras and a kinase cascade that includes Raf, Mek and MAPK. Activation of this cascade is positively regulated by a number of proteins such as KSR (kinase suppressor of Ras), SUR-8/SOC-2, SUR-6/PP2A-B and CDF-1. We describe the functional characterization of *sur-7* and several genes that regulate signaling downstream of *ras*. We identified *sur-7* by isolating a mutation that suppresses an activated *ras* allele, and showed that SUR-7 is a divergent member of the cation diffusion facilitator family of heavy metal ion transporters that is probably localized to the endoplasmic reticulum membrane and regulates cellular Zn²⁺ concentrations. Genetic double mutant analyses suggest that the SUR-7-mediated effect is not a general toxic response. Instead, Zn²⁺ ions target a specific step of the pathway, probably regulation of the scaffolding protein KSR. Biochemical analysis in mammalian cells indicates that high Zn²⁺ concentration causes a dramatic increase of KSR phosphorylation. Genetic analysis also indicates that PP2A phosphatase and PAR-1 kinase act downstream of Raf to positively and negatively regulate KSR activity, respectively.

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Introduction

The small GTPase Ras is an important regulator of eukaryotic cell proliferation and differentiation. Genetic and biochemical studies have defined a well-characterized linear pathway in which Ras functions as a molecular switch for transducing extracellular signals to the nucleus via activation of a cano-

nical mitogen-activated protein kinase (MAPK) cascade (reviewed in Vojtek and Der, 1998). Continued genetic and biochemical studies argue that multiple regulatory inputs converge on the RTK/Ras/MAP kinase cascade to ensure appropriate levels, timing and location of the signaling activity mediated by this pathway.

Genetic modifier screens in both *Drosophila* and *Caenorhabditis elegans* have identified several key players in the central linear pathway. Such screens also identified both positive and negative regulators of Ras-mediated signaling, including a number of factors that appear to regulate Raf and MEK activities. For example, KSR (kinase suppressor of Ras) was identified as a positive regulator of Ras signaling in both *C. elegans* and *Drosophila* suppressor screens (Kornfeld *et al*, 1995; Sundaram and Han, 1995; Therrien *et al*, 1995). Subsequent analysis suggested that kinase activity is not required for KSR function and that it may serve as a scaffold for Raf, MEK and other proteins (Morrison, 2001). The positive regulators *sur-6* and *sur-8* (suppressor of *ras* mutations) were identified in *C. elegans* (Sieburth *et al*, 1998, 1999). SUR-6 is a regulatory B/PR55 subunit of protein phosphatase 2A while SUR-8 is a novel but conserved leucine-rich repeat protein that complexes with Ras and Raf to enhance Raf activation (Li *et al*, 2000). Additionally, a *C. elegans* cation diffusion facilitator (CDF) family member, *cdf-1*, was identified as a positive regulator of Ras signaling (Bruinsma *et al*, 2002). Presumably, *cdf-1(lf)* results in increased cytosolic zinc concentrations, negatively affecting the pathway downstream of *let-60*/Ras and upstream of *mek-2*. There are some conflicting genetic and biochemical data however for some positive regulators of Ras signaling. Biochemical analyses indicated that KSR acts as a scaffold protein that facilitates the activation of MEK by Raf (reviewed by Morrison, 2001; Raabe and Rapp, 2002). However, previous genetic analyses in *Drosophila* and *C. elegans* suggested that KSR functions upstream of Raf in concert with SUR-6 (Therrien *et al*, 1995; Sieburth *et al*, 1999).

Here we characterize the role of a divergent CDF family member, *sur-7*, as a positive regulator of Ras signaling. Our analyses of interactions between *sur-7* and other regulators clarify the discrepancies between genetic and biochemical studies of KSR and *sur-6*, and more precisely address where within the pathway intracellular zinc may exert its effect.

Results

***sur-7(ku119)* suppresses an activated *ras* allele**

Previous studies have shown that the *C. elegans let-60/Ras* gene plays critical roles in a number of developmental processes (reviewed by Sternberg and Han, 1998). The best understood role for *let-60* is its requirement for appropriate induction of a subset of hypodermal cells to generate the

*Corresponding author. Department of Molecular, Cellular and Developmental Biology, Howard Hughes Medical Institution, University of Colorado, Boulder, CO 80309, USA. Tel.: +1 303 735 0375; Fax: +1 303 735 0175; E-mail: mhan@colorado.edu

³Present address: Howard Hughes Medical Institute, Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706, USA

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vulva, the hermaphrodite egg-laying and copulatory structure (reviewed in Kornfeld, 1997; Sternberg and Han, 1998). Reduction of *let-60* activity by mutations leads to a failure in vulva precursor cell (VPC) induction, while gain-of-function (*gf*) alleles result in VPC overinduction causing a multivulva (*Muv*) phenotype characterized by numerous ventral protrusions along the length of the animal. To identify genes acting downstream of *let-60/Ras*, screens were previously carried out in our laboratory and others to isolate alleles that suppress this *Muv* phenotype (e.g. Wu and Han, 1994; Kornfeld *et al*, 1995; Sieburth *et al*, 1998). One allele, *ku119*, was identified and mapped genetically to the right arm of LG X.

In wild-type animals, three of six VPCs are induced to a vulva fate. *let-60(gf)* mutant animals are 76% *Muv* and on average 4.6 VPCs are induced. When *ku119* is homozygous in a *let-60(n1046)* background, the *Muv* percentage and induction are reduced to nearly wild-type levels (Table I). In an otherwise wild-type background however, *ku119* exhibits no obvious vulva underinduction defects. The locus defined by *ku119* was named *sur-7*.

To determine if *ku119* is a loss-of-function mutation, we performed RNAi against *sur-7* in wild-type, *let-60(n1046)* and *let-60(n1046);sur-7(ku119)* animals (Materials and methods). RNAi against *sur-7* caused no overt phenotype in wild-type or *let-60(n1046);sur-7(ku119)* animals and strongly suppressed the *Muv* phenotype to 3.2% ($n=947$) in *let-60(n1046)*. As there were no obvious phenotypic differences between *sur-7(ku119)* and *sur-7(RNAi)*, we conclude that *sur-7(ku119)* is a strong *lf* allele.

SUR-7 cloning and classification as a CDF family member

To identify the *sur-7* locus, *ku119* was mapped using genetic markers and single-nucleotide polymorphisms (SNPs) to an LGX map position of approximately 23.9 between the SNPs *pk6169* and *F38E9.1* (Materials and methods). One cosmid in this interval (*F01G12*) and a subclone containing the single open reading frame *F01G12.2* conferred robust rescuing activity to *sur-7(ku119)*. The predicted *F01G12.2* ORF was sequenced and a G to A transition was identified at position +5 in the 5' splice site in the third of six introns (Figure 1). We performed RT-PCR to confirm the gene structure and that the *ku119* mutation results in intron 3 splicing failure. Sequence analysis of wild-type RT-PCR product confirmed that the predicted *F01G12.2* gene structure is correct. Using an exon three specific primer, we identified two RT-PCR products in *ku119* animals, one containing the third intron, which would result in premature translational stop, while the second corresponded to the predicted wild-type mRNA. Thus, *sur-7(ku119)* may not be a complete *lf* allele.

An NCBI BLAST search using the SUR-7 protein sequence produced no significant homology. However, motif analysis using the PROSITE database suggested similarity to the CDF family of proteins (Falquet *et al*, 2002). This similarity was primarily recognized due to the presence of six transmembrane domains, a feature common to most CDF proteins. CDF proteins are a class of ubiquitous metal transporters that facilitate metal ion traffic across the plasma membrane and the membranes of organelles. Another *C. elegans* CDF family member, *cdf-1*, was recently identified as a positive regulator of Ras signaling (Bruinsma *et al*, 2002). *cdf-1(lf)* alleles similarly suppress *let-60(n1046)* *Muv* to nearly wild-type levels.

Table I Double and triple mutant analyses of *sur-7* and other genes for their roles in vulval induction

Genotype	%M UV (n)	Average #VPC induced (n)
<i>let-60(n1046)</i>	76 (128)	4.62 (27)
<i>let-60(n1046);sur-7(ku119)</i>	2 (634)	3.03 (38)
<i>let-60(kuls12)</i>	86 (22)	4.68 (22)
<i>let-60(kuls12);sur-7(ku119)</i>	17 (35)	3.21 (35)
<i>lin-15(n765)</i>	91 (47)	5.37 (51)
<i>lin-15(n765) sur-7(ku119)</i>	41 (49)	3.36 (37)
<i>lin-31(n301)</i>	90 (49)	3.81 (20)
<i>lin-31(n301);sur-7(ku119)</i>	95 (65)	3.96 (20)
<i>lin-1(ar147)</i>	100 (>100)	5.91 (21)
<i>lin-1(ar147);sur-7(ku119)</i>	100 (>100)	5.85 (27)
<i>Hsp-dRaf(gf)</i>	35 (60)	3.84 (30)
<i>Hsp-dRaf(gf);sur-7(ku119)</i>	35 (62)	3.63 (28)
<i>Hsp-dRaf(gf)^a</i>	47 (30)	3.57 (30)
<i>Hsp-dRaf(gf);sur-6(ku123)^a</i>	47 (17)	3.75 (17)
<i>Hsp-dRaf(gf);sur-8(ku167)^b</i>	ND	3.75 (28)
<i>Hsp-dRaf(gf);ksr-1(ku68)^a</i>	46 (28)	3.51 (28)
<i>Hsp-dRaf(gf);mpk-1(ku1)^b</i>	ND	2.97 (28)
<i>lin-45(gf)</i>	77 (334)	4.44 (20)
<i>lin-45(gf);sur-7(ku119)</i>	2 (435)	3.06 (20)
<i>lin-45(gf);sur-8(ku167)</i>	70 (246)	3.81 (25)
<i>Sur-6(ku123) lin-45(gf)</i>	0 (334)	3.00 (23)
<i>lin-45(gf);ksr-1(ku68)</i>	1 (98)	3.00 (25)
<i>Mek-2(ku114);lin-45(gf)</i>	0 (104)	3.00 (25)
<i>Hsp-mpk-1(gf) hsp-Dmek(gf)</i>	97 (240)	ND
<i>Hsp-mpk-1(gf) hsp-Dmek(gf);sur-7(ku119)</i>	95 (273)	ND
<i>Hsp-mpk-1(gf) hsp-Dmek(gf);sur-2(lf)^c</i>	0 (25)	ND
<i>Rol-4(sc8); let-60(n1046)</i>	86 (217)	ND
<i>Par-1(b274);let-60(n1046)</i>	100 (112)	ND
<i>Sur-6(ku123);let-60(n1046)</i>	6 (240)	ND
<i>Sur-6(ku123);par-1(b274);let-60(gf)</i>	65 (48)	ND
<i>let-60(n1046);par-1(b274);sur-7(ku119)</i>	94 (54)	ND
<i>Sur-8(ku167) let-60(n1046)</i>	4 (333)	ND
<i>Sur-8(ku167) let-60(n1046);par-1(b274)</i>	65 (111)	ND
<i>let-60(n1046);ksr-1(ku68)</i>	0 (>100)	ND
<i>let-60(n1046);par-1(b274);ksr-1(ku68)</i>	13 (23)	ND
<i>Mpk-1(ku1);let-60(n1046)</i>	0 (>100)	ND
<i>Mpk-1(ku1);let-60(n1046);par-1(b274)</i>	2 (41)	ND

%Muv, percentage of worms with a Multivulva phenotype examined under a dissecting scope. #VPC induced, number of vulval precursor cells induced to vulval cells examined under Nomarski optics on a compound microscope. (n), number of animals examined. ND, not determined. *kuls12* is an integrated array carrying multiple copies of wild-type *let-60*. For *lin-15(n765)* experiments, animals were grown at 18.2°C. *hsp-dRaf(gf)* is described in Materials and methods and Sieburth *et al*, (1999). *lin-45(gf)* is described in the text and Materials and methods. The *hsp-mek/mpk-1(gf)* strain has an integrated array containing gain-of-function mutant genes of both *mpk-1* and *d-mek* (Lackner and Kim, 1998) (see Materials and methods for assay conditions. *par-1* was marked with *rol-4* in all lines tested. *sur-8*, *sur-6* and *ksr-1* were marked with *dpy-20*, *unc-29* and *lon-2*, respectively. *sur-7(ku119)* and *mek-2(ku114)* homozygotes were identified by sequence analysis. Induction data were not collected for *par-1* experiments in order to monitor embryonic phenotype to score for homozygous *par-1(lf)*.

^aSieburth *et al* (1999).

^bSieburth and Han (1998).

^cLackner and Kim (1996).

CDF proteins are found in both prokaryotes, and eukaryotes and vary greatly in size and sequence. In general, CDF proteins possess six transmembrane domains and a histidine-rich loop between membrane spanners IV and V (Palmiter



Figure 1 SUR-7 is a cation diffusion facilitator protein. (A) Structure of *sur-7* and lesion identified in *sur-7(ku119)*. A G to A transition was identified in the 5' splice site of the third intron. (B) Comparison between SUR-7 and a consensus for the CDF family of proteins. SUR-7 contains six transmembrane domains (shaded regions). Five of six domains align physically with those in other CDF proteins. Most CDF proteins have a histidine-rich loop with as many as 13 histidine residues between TMs IV and V. SUR-7 has a short H-rich region between TMs III and IV. (C) Alignment between SUR-7, CDF-1 and *S. pombe* Zhf-1. SUR-7 shares the greatest degree of identity with other CDF proteins from TM VI to its C-terminus. In this region, SUR-7 is 16% identical and 36% similar to CDF-1, and 17% identical and 32% similar to Zhf1. CDF-1 and Zhf1 are 24% identical and 44% similar over the same region. Black backgrounds represent identity and outlines represent similarity.

and Findley, 1995; Paulsen and Saier, 1997). This H-rich loop, thought to be important for metal ion binding, and the termini of the protein presumably face cytoplasmically. There are several examples of deviation from these family characteristics. Yeast Msc2 and human hZTL1 have 12 membrane spanners (Li and Kaplan, 2001; Cragg *et al*, 2002). Additionally, the termini of hZTL1 are predicted to face extracellularly and hZnt6 has no H-rich loop (Huang *et al*, 2002). Therefore, this is a loosely defined family of proteins, which share the one specific trait of conferring either resistance or hypersensitivity to various heavy metals such as Zn^{2+} , Cd^{2+} , Cu^{2+} , Co^{2+} and Ni^{2+} when their expression is altered (Gaither and Eide, 2001). SUR-7 has no H-rich loop between membrane spanners IV and V. Instead there is a cluster of five histidine residues

between spanners III and IV (Figure 1). We compared the sequences of five representative CDF proteins (yeast cot1, yeast zhf1, human Znt1, human Znt5 and *C. elegans* CDF-1) and found the greatest sequence similarities in their C-termini and six transmembrane domains. SUR-7 displays the greatest sequence similarity to these proteins from membrane spanner VI through the C-terminus, with sequence identities ranging from 16 to 23% (Figure 1). While SUR-7 lacks similarity to other CDF family members in the remainder of its sequence, the N-terminal half of SUR-7 and the other proteins primarily define the other five transmembrane domains. When aligned against the other five CDF proteins, all but one of the predicted SUR-7 membrane spanners aligned precisely with those of the other protein family members.

***sur-7(ku119)* animals are sensitive to high Zn²⁺ concentrations**

Like several human and yeast CDF proteins, mutations in *C. elegans cdf1* caused hypersensitivity toward increased Zn²⁺ concentrations (Palmiter and Findley, 1995; Bruinsma *et al*, 2002). To investigate a similar response by *sur-7(ku119)* animals, we performed a Zn²⁺ sensitivity assay (Bruinsma *et al*, 2002; Materials and methods). Expectedly, *sur-7(ku119)* animals were more sensitive to increasing concentrations of Zn²⁺ than wild type (Figure 2). The progeny of animals placed on metal-supplemented growth media showed increasing sensitivity to Zn²⁺ concentrations at and above 0.25 mM as assayed by their rate of developmental maturation. These differences were found to be significant at and above 1.0 mM Zn²⁺ (student's *t*-test: α 0.05). However, *sur-7(ku119)* animals were more tolerant to increased Zn²⁺ than *cdf-1(n2527)* controls. The difference in tolerance between *cdf-1(n2527)* and wild type as well as *sur-7(ku119)* was significant at 0.25 and 0.05 mM Zn²⁺ (Tukey–Kramer HSD: α 0.05); however, Zn²⁺ tolerance was not statistically different between *sur-7(ku119)* and *cdf-1(n2527)* at and above 1.0 mM Zn²⁺. While these observations may reflect functional differences between the two proteins, they suggest that like CDF-1, SUR-7 functions as a heavy metal ion transporter, and specifically, but perhaps not exclusively, mediates cytoplasmic efflux of Zn²⁺ ions. As further evidence for similar functions of SUR-7 and CDF-1, we found that alleles in both genes conferred statistically significant (student's *t*-test: α 0.05) increased tolerance—rather than heightened sensitivity as was the case for Zn²⁺—to Cu²⁺ (at 0.5 mM but not above) compared to wild-type controls, but showed little or no difference in sensitivity for other heavy metal ions tested (Co²⁺ and Cd²⁺) (Materials and methods; data not shown). CDF proteins are not thought to function as copper transporters; therefore, the increased resistance to Cu²⁺ levels exhibited by both *sur-7(ku119)* and *cdf-1(n2527)* may reflect physiological conditions resulting from excess Zn²⁺ accumulation. Nonetheless, these results support the view that SUR-7

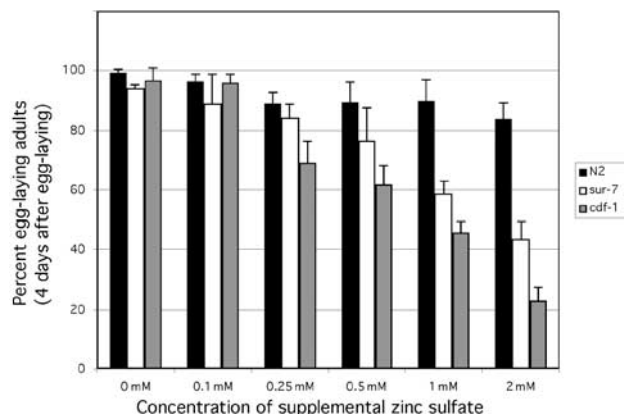


Figure 2 *cdf-1(lf)* and *sur-7(lf)* mutants display heightened toxicity toward increasing concentrations of Zn²⁺. Animals grown on metal supplemented media were scored for percentage reaching egg-laying maturity after 4 days (Materials and methods). Each bar represents the mean of three independent experiments and shows the calculated standard deviation. *sur-7(ku119)* and *cdf-1(n2527)* responses were found to be significantly different from wild type (but not one another) at and above 1.0 mM Zn²⁺ (Tukey–Kramer: α 0.05).

and CDF-1 similarly affect heavy metal ion homeostasis in *C. elegans*.

***SUR-7* is likely localized to the endoplasmic reticulum membrane**

We next generated a translational green fluorescent protein (GFP) fusion construct to determine the expression pattern and subcellular distribution of SUR-7 (Materials and methods). The *sur-7::GFP* construct is functional since the expression of this construct rescues *sur-7(ku119);let-60(n1046)* (data not shown). We integrated an extrachromosomal array carrying this construct into the genome. SUR-7::GFP was expressed in most tissues with the notable exception of intestinal cells. Under a UV-light dissecting microscope, GFP was seen to be strongly expressed in the pharynx, some tail cells and the vulva (data not shown). Under higher magnification, GFP was observed in many if not all neurons, muscles and hypodermal cells. Strikingly, SUR-7::GFP was not localized to the plasma membrane, as was the case for a CDF-1::GFP construct (Bruinsma *et al*, 2002), but was enriched at the nuclear periphery and throughout the cytoplasm in a fenestrated pattern (Figure 3A and B). We suggest that this localization is endoplasmic reticular for two reasons. First, this expression pattern is indistinguishable from that of a *C. elegans* YFP::TRAM construct (data not shown; TRAM is an endoplasmic reticulum (ER) transmembrane protein) (Rolls *et al*, 2002). Secondly, several CDF family members are known to be ER localized (Li and Kaplan, 2001; Clemens *et al*, 2002). We suggest that rather than functioning to transport heavy metals extracellularly like CDF-1, SUR-7 is localized to the membrane of cellular organelles, likely the ER, and functions to sequester intracellular Zn²⁺ into this structure, likewise reducing cytoplasmic levels of this metal ion. The two proteins function redundantly however, as RNAi of *sur-7* in the *cdf-1(lf)* mutant background resulted in slow

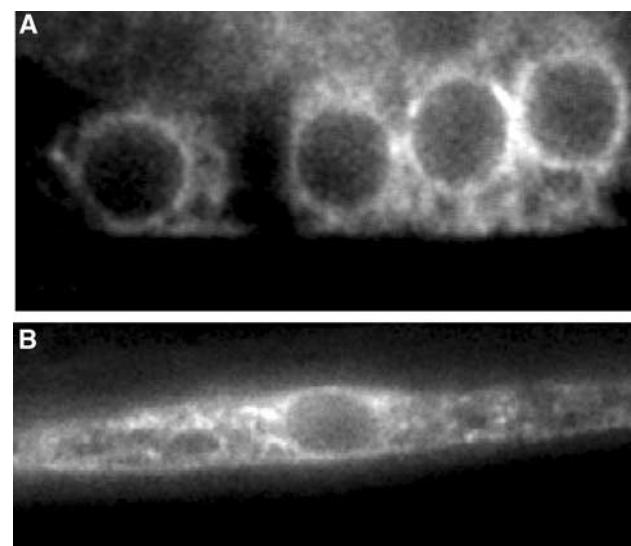


Figure 3 A functional translational SUR-7::GFP construct is expressed in most tissues and is probably localized to the ER. (A) High-powered magnification of SUR-7::GFP in four descendents of the vulva precursor cell P6.p and (B) a body wall muscle cell. SUR-7::GFP is enriched at the nuclear envelope and expressed in a reticular pattern throughout the cytoplasm.

growth and pleiotropic developmental defects (data not shown).

sur-7, ksr-1 and sur-6 likely act on factors downstream of Raf

The observations that loss-of-function mutations in *sur-7*, *ksr-1* and *sur-6* suppress activated *ras* suggest that these genes act on factors downstream of *ras*. To understand the mechanism of their functions, it is critical to know whether they also act downstream of *raf*. Previous genetic analyses of *sur-6*, *sur-8* and *ksr-1* suggested that each functions upstream of *lin-45/Raf*, as *lf* mutations in none of these three genes can suppress the Muv phenotype associated with an overexpressed *raf* gain-of-function (*gf*) construct (Sieburth et al, 1998, 1999) (Table I). Similarly, a *Drosophila ksr* *lf* allele could not suppress the rough eye phenotype caused by overexpression of the same Raf construct (Therrien et al, 1995). This *raf(gf)* construct contains a *Drosophila raf* transcript fused to a transmembrane domain of the Torso receptor and is under transcriptional control of a heat shock promoter (Dickson et al, 1992). Like mutations in the other positive regulators, *sur-7(ku119)* could not suppress the Muv phenotype associated with this construct (Table I). However, biochemical work has indicated that KSR directly interacts with both Raf and MEK and therefore *ksr-1* should function genetically downstream of *lin-45/Raf*. These conflicting observations have been puzzling and have raised concerns about assays using this *raf(gf)* construct. To address this concern, we took advantage of another, likely more physiologically relevant, *raf(gf)* construct. The phosphorylation state of Raf is a major mechanism of its activity modulation (Brtva et al, 1995; Hu et al, 1995; Chong et al, 2001). Basal phosphorylation of putative 14-3-3 binding sites is inhibitory to Raf activity. Relief of this inhibition may be mediated by protein phosphatases 1 and 2A (Abraham et al, 2000; Jaumot and Hancock, 2001). Two consensus 14-3-3 binding sites were identified in LIN-45, homologous to those in vertebrate B-Raf, and were shown to inhibit LIN-45 activity when phosphorylated (Chong et al, 2001). Furthermore, expression of a *lin-45* construct encoding alanine substitutions for these conservative serine residues caused a penetrant Muv phenotype in wild-type worms. We integrated this *lin-45(gf)* construct producing a strain with a strong Muv phenotype (Materials and methods and Table I). In contrast to observations using *hsp-dRaf(gf)*, we observed that both *sur-6(lf)* and *ksr-1(lf)*, but not *sur-8(lf)*, robustly suppressed the Muv phenotype caused by the *lin-45(gf)* construct (Table I). These data support a model in which SUR-8 exerts its positive regulation by facilitating the interaction between Ras and Raf (Li et al, 2000) while SUR-6 and KSR-1 function downstream of Raf possibly in a common convergent regulatory event (Sieburth et al, 1999). Moreover, PP2A has been shown to associate with Raf-1 in vertebrate cell culture and potentially mediate dephosphorylation of S259 (a putative 14-3-3 binding site), thereby contributing to Raf activation (Abraham et al, 2000). Our results suggest that such potential regulation of Raf by PP2A at S259 is not mediated by the SUR-6/PR55 regulatory subunit, since *sur-6(lf)* can suppress LIN-45 activation caused by mimicking dephosphorylation of this residue, which is presumably targeted by PP2A. Lastly, our data argue that *sur-7* exerts its influence downstream of *lin-45* as *sur-7(ku119)* also suppresses *lin-45(gf)* (Table I).

Our genetic analysis also indicates that intercellular zinc concentration, regulated by SUR-7 activity, likely affects this pathway upstream of MEK. *sur-7(ku119)* does not suppress the Muv phenotype of a previously described strain containing gain-of-function mutant genes in both *mpk-1* and the *Drosophila mek* gene (Lackner and Kim, 1998) (Table I). The Muv phenotype of this *mpk-1(gf)/Dmek(gf)* strain has been shown to be epistatic to mutations in genes acting upstream of *mek* but suppressed by mutations in factors known to act downstream of *mpk-1* (Lackner and Kim, 1998). Consistent with SUR-7 acting upstream of MEK, *ku-119* also failed to suppress the Muv phenotype of loss-of-function mutations in *lin-1* and *lin-31*, two transcription factors that are targets of MAPK in the VPCs. These observations are consistent with the study of CDF-1 in *Xenopus oocytes* (Bruinsma et al, 2002). Together, these results suggest that Zn ions negatively affect this signaling pathway at the level of Raf activation of MEK. Protein interaction analyses and the data we present here place SUR-6 and KSR-1 as the only other known positive regulators at this point in the pathway.

sur-7 and sur-6 antagonize par-1 in regulating ksr activity

The kinase C-TAK1 (Cdc-25C-associated kinase 1) has recently been shown to act as a negative regulator of Ras signaling through the MAPK cascade (Muller et al, 2001). C-TAK1 was shown to phosphorylate murine KSR and inhibit its translocation to the plasma membrane. This prevents KSR from associating with activated Raf, thereby abrogating transduction of the signal to MEK and MAPK. The *C. elegans* homolog of C-TAK1 is PAR-1, a kinase previously defined for its role in early embryonic cell polarity (Guo and Kemphues, 1995). No role for *C. elegans par-1* in Ras-mediated VPC induction has previously been described, but mutations in *par-1* have recently been shown to alter vulval morphology (Hurd and Kemphues, 2003). Thus, we investigated a role for *par-1* in Ras-mediated VPC induction. *par-1* *lf* mutant animals exhibit a 100% maternal embryonic lethal phenotype as a result of failed partitioning of early embryonic cytoplasmic determinants. We used a balanced *par-1(b274)* strain to generate *par-1(b274);let-60(n1046)* double mutants as well as triple mutants containing *lf* in various positive regulators. The *par-1(b274);let-60(n1046)* strain exhibited a 100% Muv phenotype (compared to 76% for *let-60(n1046)* alone) (Table I). This increase in Muv percentage suggests that PAR-1 does play a negative role in VPC induction and may function similarly to its murine homolog by regulating KSR-1. If this is true, then *par-1(lf)* may revert the suppression of the *ras(gf)* phenotype by positive regulators upstream of *ksr-1* but not regulators downstream. Indeed, this is what we observed (Table I). We used *sur-8* and *mpk-1* as controls since genetic characterization of these genes placed them respectively upstream and downstream of *lin-45/Raf*. An *mpk-1* partial *lf* allele robustly suppressed the Muv phenotype of *let-60(n1046)* in the presence of *par-1(b274)*; however, *sur-8(ku167)* suppression was overridden and the resulting triple mutant was 65% Muv. The suggested role for *par-1* also predicts that *ksr-1(lf)* should still suppress *let-60(n1046)* with *par-1(b274)* in the background. Again, this is what we observed. Importantly, neither *sur-6(lf)* nor *sur-7(lf)* could suppress *let-60(n1046)* with *par-1(b274)* in the background, suggesting that the loss-of-function mutant

phenotype of *par-1* is epistatic to that of both *sur-6* and *sur-7*. Given that PAR-1 most likely acts upstream of KSR based on biochemical data on the mammalian homologs (Muller *et al*, 2001) and genetic epistatic relationships between them (Table I), this result also suggests that SUR-6 and SUR-7 act upstream of KSR-1 to regulate the signaling activity.

High Zn²⁺ levels increase KSR phosphorylation in mammalian tissue culture cells

In mammalian tissue culture cells, C-TAK1 negatively regulates KSR function by phosphorylating a serine residue at 392 (Muller *et al*, 2001). Such a modification results in KSR binding to 14-3-3 and being cytoplasmically sequestered. Our genetic data presented above suggest that increased Zn²⁺ concentrations in *sur-7(ku119)* animals may negatively affect signaling through the MAPK cascade by one of at least three means. Increased Zn²⁺ levels may enhance the activity of PAR-1/C-TAK1, reduce the activity of PP2A that is regulated by the B-subunit SUR-6, or reduce KSR activity via a mechanism that is independent of PAR-1 and SUR-6. To further address these possibilities, we examined the effect of increased Zn²⁺ concentration on KSR phosphorylation in HEK 293 cells. We observed a striking shift in mobility of KSR after treating the cell culture with 1 mM Zn²⁺ for 3 h (Figure 4A). This shift was due to phosphorylation of the protein since it is eliminated by treatment with lambda phosphatase (Figure 4B). Interestingly, this mobility shift of

KSR is not obviously affected by alanine substitutions at S297 and S392, the two major phosphorylation sites in quiescent cells (Cacace *et al*, 1999; Volle *et al*, 1999). Since S392 is the C-TAK1 phosphorylation target, our results suggest that elevated Zn²⁺ concentrations do not promote KSR phosphorylation by affecting C-TAK1 activity on S392. However, we cannot exclude the possibility that elevated Zn²⁺ concentrations act on C-TAK1 for phosphorylation at different residues of KSR.

We also observed that Zn²⁺-induced KSR phosphorylation was not obviously affected by the addition of EGF to the medium (Figure 4A). Nor was this phosphorylation altered by addition of a MEK inhibitor (PD98059) (Figure 4C). These results suggest that the Zn²⁺-induced phosphorylation of KSR is neither inhibited by activation of Ras nor mediated by an ERK feedback activity. Furthermore, phosphatase inhibitors (okadaic acid or calyculin A) did not mimic or cause an additional up-shift of KSR mobility (Figure 4C; data not shown), suggesting that it is unlikely that Zn²⁺ acts to inhibit a serine/threonine phosphatase like PP2A. In addition, we have shown that the observed effect on KSR phosphorylation by heavy metal ions is likely Zn²⁺ specific, because the addition of high concentrations of Co²⁺, Cu²⁺, Mg²⁺ or Ca²⁺ did not lead to an up-shift of KSR mobility (Figure 4D, data not shown).

Discussion

Our observations lead us to propose an updated model for the functions of a number of regulators in Ras signaling during vulva induction in *C. elegans* (Figure 5). SUR-8 functions to facilitate the activation of Raf by Ras, a role that is consistent with the direct interactions between SUR-8 and Ras and SUR-8 and Raf (Sieburth *et al*, 1998; Li *et al*, 2000), as well as our new result that an activated *lin-45/Raf* is epistatic to loss of *sur-8*. Conflicting genetic and biochemical data concerning *ksr-1* and *sur-6* are resolved, as mutations in both have now been shown to be epistatic to activated *lin-45/Raf*. These genetic observations for KSR are consistent with biochemical studies indicating that KSR acts as a scaffolding protein to facilitate activation of MEK by Raf (Roy *et al*, 2002). The

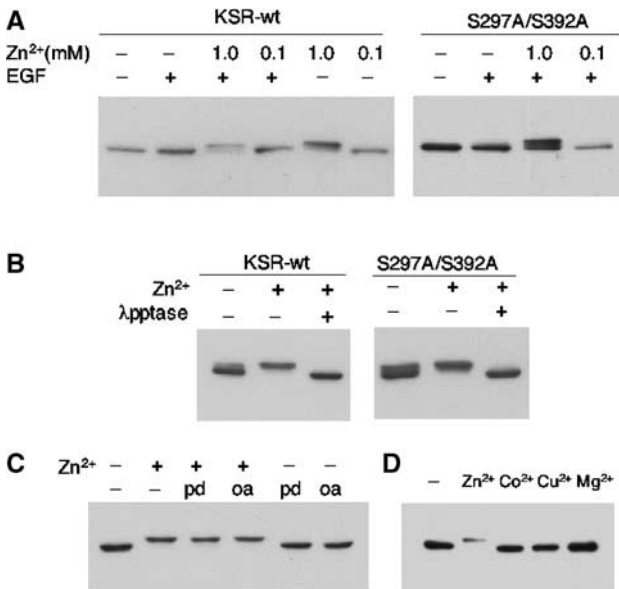


Figure 4 Elevated Zn²⁺ levels drastically increase the phosphorylation of KSR in HEK293 cells. (A) Addition of 1 mM Zn²⁺ causes a mobility shift of KSR. Flag-tagged KSR1 was transfected into HEK293 cells. At 24 h after transfection, cell cultures were treated with ZnSO₄ for 3 h and/or 50 ng/ml EGF for 3 min. The mouse KSR protein in the cell lysates was analyzed by SDS-PAGE and Western blot using anti-M2 antibody. In a control test, a Flag-tagged Smad3 protein displayed no shift under the same condition (data not shown). On the right panel, both serine 297 and 392 were changed to alanine by *in vitro* mutagenesis. (B) *In vitro* treatment of lambda phosphatase eliminated the up-shift of KSR, indicating that the Zn²⁺-induced shift of the protein is due to phosphorylation. (C) Phosphatase inhibitors did not mimic or cause additional up-shifting of KSR mobility. PD, PD98059. OA, Okadaic acid. (D) Addition of 1 mM CoCl₂, CuSO₄ or MgCl₂ does not cause the up-shift of KSR mobility.

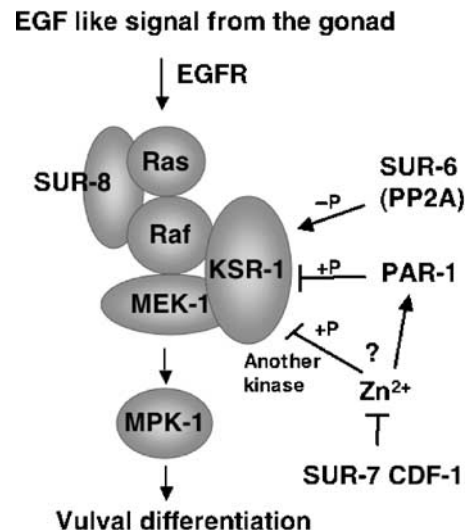


Figure 5 A model for the roles of several regulators of Ras-mediated vulval induction in *C. elegans*. See text for description.

genetic data suggesting that *ksr-1* and *sur-6* function in a common regulatory event (Sieburth et al, 1999) are also supported by our findings. The fact that *sur-6(lf)* suppresses a *lin-45(gf)* mutant that mimics dephosphorylation of a potential PP2A target residue suggests that SUR-6/PR55 does not mediate dephosphorylation of this LIN-45/Raf residue alone. Thus, our results argue for another role for PP2A, mediated by the SUR-6/PR55 subunit. One attractive model would predict that SUR-6 mediates dephosphorylation of the inhibitory effect by PAR-1/C-TAK1 on KSR-1. Indeed, the mammalian homolog of SUR-6 was recently shown to associate with KSR-1 in a PDGF-stimulated-dependent manner, and dephosphorylation of the C-TAK1 target residue as well as KSR1 membrane targeting were shown to require PP2A activity (Ory et al, 2003). Finally, the genetic and biochemical data are also consistent with the suggestion that Zn^{2+} ions also act downstream of *lin-45/Raf* but either upstream or parallel and antagonistic to *par-1*. We thus suggest that increased intracellular Zn^{2+} , a likely result of loss of *sur-7* function, inhibits KSR activity by either promoting phosphorylation of the protein by another kinase or by C-TAK1 at sites other than S297 and S392.

At present, it is not totally clear why the expression of the previously used *hsp-dRaf(gf)* construct resulted in constitutive signaling activity that is independent of KSR function in both *Drosophila* and *C. elegans*. As mentioned in the text, this *raf(gf)* construct contains a *Drosophila raf* transcript fused to a transmembrane domain of the Torso receptor (Dickson et al, 1992). The prevailing model suggests that KSR facilitates interaction between Raf and Mek at the plasma membrane upon activation of Ras (Morrison, 2001). The Raf-Torso fusion protein, which is constitutively targeted to the plasma membrane due to the transmembrane domain of Torso, may alter the conformation of Raf in such a way that it drastically increases its affinity for Mek without a requirement for KSR. Nevertheless, since dephosphorylation of the two 14-3-3 binding sites of B-raf was clearly shown to be critical for Raf activation (Chong et al, 2001), the alanine substitutions at these two sites in Raf provide a gain-of-function mutant gene that appears to simply render the protein, to a large extent, independent of upstream activation, making it ideal for genetic epistasis analysis.

Why should the cellular concentration of Zn^{2+} have such a specific effect on Ras signaling? While proper levels of Zn^{2+} may have an important influence on general developmental decisions, an attractive model is that the Ras-mediated cell-signaling pathway is used to sense and regulate the cellular levels of Zn^{2+} and/or other heavy metal ions. For example, a target of MAPK may be involved in regulating the activity or transcription of ion transporters to complete feedback regulation of cellular levels of these ions. This in turn would downregulate Ras-mediated signaling. At least one other heavy metal ion transporter, when its function is reduced, can affect signaling through the Ras/MAPK pathway. We blasted the Wormbase *C. elegans* protein database with the CDF-1 protein sequence. Numerous worm proteins were identified as potential members of this family, and we performed RNAi against the four most similar proteins (Y105E8A.3, T18D3.3, Y39E4A.2 and ZC395.3). Interestingly, only RNAi against the gene ZC395.3 could suppress the Muv phenotype of *let-60/ras(gf)* (27.9% Muv). This gene encodes the least similar of the four proteins to CDF-1, and

has previously been given the name *toc-1* (transporter of divalent cations) (Ewbank et al, 1997). It will be important to address if increased production of zinc transporters or activity of these proteins is modulated in response to increased or hyperactive signaling through the Ras/MAPK cascade.

Materials and methods

C. elegans strains and genetics

Mutants were derived from the wild-type Bristol N2 strain and cultured under standard conditions at 20°C unless otherwise noted (Brenner, 1974). Some strains used for mapping and genetic analysis were obtained from the *Caenorhabditis* Genetic Center in Minnesota.

sur-7 analysis and RNAi

sur-7(ku119) was isolated as a recessive suppressor of the *let-60(n1046)* Muv phenotype as previously described (Sundaram and Han, 1995). *let-60(n1046); sur-7(ku119)* was outcrossed five times, creating the strains MH664 [*let-60(n1046);sur-7(ku119)*] and MH801 [*sur-7(ku119)*] used for the majority of genetic analyses. *sur-7* was genetically mapped to the right arm of LGX between *lin-15* and *sup-10* using standard three-factor mapping. For SNP mapping, the strain *lin-15(n765ts) sur-7(ku119) sup-10(n983)* was generated. This triple mutant strain was then mated to the Hawaiian wild-type isolate CB4856 to generate *trans*-heterozygous progeny. These animals were allowed to self and the resulting progeny were then scored for either *lin-15-non-sup-10* or *sup-10-non-lin-15* recombinants. Nine SNPs in the *lin-15 sup-10* interval were analyzed to determine the positions of recombination events (Koch et al, 2000), and each recombinant was tested for its ability to suppress *let-60(n1046)* and therefore the presence of wild type or mutant *sur-7*. Three informative recombinants narrowed the *sur-7* locus to a two-cosmid region (F01G12 and T24D11) between PK6169 and F38E9.1. The cosmids were injected into MH664 [*let-60(n1046);sur-7(ku119)*] animals at 15 µg/ml along with 100 µg/ml *sur-5::GFP*, which served as a marker for transgenic progeny (Gu et al, 1998). F01G12 was found to rescue the suppression of the Muv phenotype to >50% Muv in two of six transgenic lines generated. pJHY21 is an XbaI/SalI digested F01G12 fragment cloned into pBluescript. This 6 kb fragment contains ~3 kb of the 5' region to the open reading frame F01G12.2 and the entire ORF followed by ~750 kb of the 3' sequence. Seven of seven transgenic lines established with this subclone rescued the *sur-7(ku119)* phenotype. pJHY21 was used to generate a full-length translation SUR-7::GFP fusion construct (pJHY26) by introducing a unique restriction site just prior to the predicted stop codon (Quickchange site-direct mutagenesis kit from Stratagene) and ligating GFP coding DNA from the vector pPD118.85 (a gift from Dr Andrew Fire).

The molecular lesion associated with *sur-7(ku119)* was identified by sequencing PCR amplification of genomic DNA from *let-60(n1046);sur-7(ku119)* and *let-60(n1046)* lysates.

The full-length EST, yk462h8 (a gift from Yuji Kohara at the National Institute of Genetics), was used to generate double-stranded RNA for *sur-7*(RNAi) experiments following standard procedures (Fire et al, 1998). RNA was injected into the intestines of young adults and their progeny scored for phenotypes.

Constructing a lin-45(gf) allele and assaying vulval differentiation using a mek(gf)/mpk(gf) strain

A *dpy-20(e1282)* strain carrying an extrachromosomal array containing a *lin-45(gf)* gene (Chong et al, 2001) behind a hypodermal specific promoter from the *col-10* gene, and the transgenic marker *sur-5::GFP* was first constructed. This strain was subjected to γ -irradiation to induce germline integration following standard procedures (Epstein and Shakes, 1995). Two integrated lines were obtained and subjected to three rounds of backcrossing before use in epistatic analyses. The integrated line *kuIs57* was found to possess a more penetrant Muv phenotype and was used for all experiments. pJHY26 (*sur-7::GFP*) was similarly integrated into a wild-type N2 strain to create the line *kuIs61*. To make certain that the suppression of the Muv phenotype of the *col-10:lin-45(gf)* transgene by *sur-7(ku119)* is not due to a suppression

of the *col-10* promoter activity, we constructed a *col-10::GFP* and introduced it into *C. elegans*. The GFP expression of this fusion construct in the *sur-7* mutant is no different from that in wild type.

A strain carrying an integrated DNA array that contains *gf* mutant copies of both the *mpk-1* and the *Drosophila mek* genes was previously described and shown to effectively cause a highly penetrant Muv phenotype (Lackner and Kim, 1998). The Muv phenotype was shown to be epistatic to Vulvaless phenotype of genes acting upstream of *mek* but suppressed by mutations in downstream transcription factors. To determine if *sur-7(ku119)* is epistatic to this Muv phenotype, a strain containing both *ku119* and this array were constructed. Adult animals growing at 20°C were allowed to lay eggs for 6 h and eggs were placed at 25°C for 48 h before scoring the Muv phenotype.

Heavy metal toxicity assays

For metal toxicity, the protocol in Bruinsma *et al* (2002) was followed. Molten nematode growth medium (NGM) was supplemented with varying concentrations of heavy metal prior to pouring on culture plates. OP50, the standard bacterial strain for culturing *C. elegans*, was then spotted on these plates and worms were cultured at 20°C according to standard protocols. L4-young adult worms were placed on metal-supplemented plates overnight and transferred to fresh plates the following day. These animals were allowed to lay eggs for 3 h and then removed. Four days later, the progeny were assayed for the percentage of egg-laying animals for Zn²⁺, Cd²⁺ and Co²⁺ sensitivity assays or the percentage of L1 arrested larvae for Cu²⁺ sensitivity assays. Zn²⁺ and Cu²⁺ assays were performed in triplicate and an average of 100 animals were scored for each data point.

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Cell culture and transfection

HEK293 cells were cultured in DMEM (Invitrogen) containing 10% FBS. Cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer's directions in serum-free DMEM. At 24 h after transfection, cells were treated with ZnSO₄ for 3 h and/or 50 ng/ml EGF for 3 min. Cells were lysed in NP-40 lysis buffer and the mouse KSR protein was analyzed by SDS-PAGE and Western blot analysis using anti-M2 antibody (Sigma). For lambda phosphatase treatment, after cells were lysed, flag-tagged KSR proteins were immunoprecipitated using anti-M2 antibody and protein-G sepharose beads. Samples were washed three times with NP-40 lysis buffer, once with 20 mM Tris buffer (pH 7.5), and then incubated with lambda phosphatase for 30 min at 30°C.

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