C. elegans SUR-6/PR55 cooperates with LET-92/protein phosphatase 2A and promotes Raf activity independently of inhibitory Akt phosphorylation sites

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Summary

Protein phosphatase 2A (PP2A) can both positively and negatively influence the Ras/Raf/MEK/ERK signaling pathway, but its relevant substrates are largely unknown. In *C. elegans*, the PR55/B regulatory subunit of PP2A, which is encoded by *sur-6*, positively regulates Ras-mediated vulval induction and acts at a step between Ras and Raf. We show that the catalytic subunit (C) of PP2A, which is encoded by *let-92*, also positively regulates vulval induction. Therefore SUR-6/PR55 and LET-92/PP2A-C probably act together to dephosphorylate a Ras pathway substrate. PP2A has been proposed to activate the Raf kinase by removing inhibitory phosphates from Ser259 from Raf-1 or from equivalent Akt phosphorylation sites in other Raf family members. However, we find that mutant forms of *C. elegans* LIN-45 RAF that lack these sites still require *sur-6*. Therefore, SUR-6 must influence Raf activity via a different mechanism. SUR-6 and KSR (kinase suppressor of Ras) function at a similar step in Raf activation but our genetic analysis suggests that KSR activity is intact in *sur-6* mutants. We identify the kinase PAR-1 as a negative regulator of vulval induction and show that it acts in opposition to SUR-6 and KSR-1. In addition to their roles in Ras signaling, SUR-6/PR55 and LET-92/PP2A-C cooperate to control mitotic progression during early embryogenesis.

Key words: PP2A, PR55, Raf, PAR-1, Vulva, *C. elegans*

Introduction

The kinase Raf is a key target of the Ras GTPase during both normal development and oncogenesis, and acts upstream of MEK and ERK in the mitogen activated protein kinase (MAPK) cascade (Morrison and Cutler, 1997). Raf activation appears to involve multiple steps, including release of autoinhibition by the Raf N terminus, recruitment to the plasma membrane, Ras-GTP binding, multimerization and changes in phosphorylation status (Chong et al., 2003). Genetic screens in *C. elegans* and *Drosophila* have identified several gene products that function at a step between Ras and Raf and therefore appear to regulate Raf activation (Sternberg and Alberola-Ila, 1998; Moghal and Sternberg, 2003). These include KSR (kinase suppressor of Ras), a Raf-related protein that binds to Raf, MEK and ERK, and may function non-catalytically as a scaffold (Morrison, 2001), SUR-8, a leucine-rich repeat protein that binds to Ras (Sieburth et al., 1998), and SUR-6, a PR55/B-regulatory subunit of protein phosphatase 2A (PP2A) (Sieburth et al., 1999). The mechanisms by which these proteins promote Raf activation are not well understood. However, the molecular nature of SUR-6 suggests that it may regulate the phosphorylation status of Raf or of another Raf regulatory protein.

PP2A is a heterotrimeric serine/threonine phosphatase composed of invariant catalytic ('C') and structural ('A') subunits and a variable regulatory subunit ('B') that directs the AC core complex to different substrates (Janssens and Goris, 2001). PP2A both positively and negatively influences the Ras/MAP kinase pathway in *Drosophila* and in mammalian cells, suggesting it may act on multiple Ras pathway substrates (Sontag et al., 1993; Alessi et al., 1995; Wassarman et al., 1996; Maixner et al., 1998; Ugi et al., 2002; Strack, 2002; Silverstein et al., 2002). Unfortunately, the pleiotropic defects caused by interfering with PP2A activity in vivo and the very broad substrate specificity of PP2A in vitro have hampered attempts to identify its most functionally relevant substrates. The finding that partial loss-of-function alleles of *sur-6*/B specifically reduce Ras signaling in *C. elegans* (Sieburth et al., 1999) provides a potentially simpler genetic model system for studying the effects of PP2A on the Ras pathway.

C. elegans vulval development is a well characterized model system for studying the Ras signaling pathway (Moghal and Sternberg, 2003). The vulva is generated by a specialized subset of ventral ectodermal blast cells called vulval precursor cells (VPCs) (Fig. 1A). During larval development, Ras signaling induces three of six equipotent VPCs to execute a vulval lineage. The remaining three uninduced VPCs execute a non-vulval hypodermal lineage. The EGF receptor/Ras/

MAP kinase pathway (Fig. 1B) is required for vulval induction, as complete loss of pathway activity causes a Vulvaless (Vul) phenotype in which no VPCs adopt vulval fates. Increased Ras activity causes a Multivulva (Muv) phenotype in which greater than three VPCs adopt vulval fates. Thus, the extent of vulval differentiation provides a sensitive readout of Ras signaling levels. Other signaling pathways, including a Wnt/β-catenin pathway, independently influence vulval fate induction and can also mutate to cause partial Vul or Muv phenotypes (Eisenmann et al., 1998; Gleason et al., 2002) (Fig. 1B).

The *sur-6* gene encodes the only PR55/B regulatory subunit of PP2A in *C. elegans* (Sieburth et al., 1999). Two partial loss-of-function missense alleles of *sur-6*, *ku123* and *cs24*, do not significantly perturb vulval development, but do strongly enhance the Vul phenotype caused by reducing Ras pathway activity (Sieburth et al., 1999). SUR-6 appears to function between (or in parallel to) Ras and Raf as *sur-6* alleles suppress the Muv phenotype caused by activated Ras but not that caused by an activated form of Raf, Torso^{4021}-D-Raf (Sieburth et al., 1999). By contrast, mutations in the downstream genes *mek-2* and *mpk-1* efficiently suppress both the activated Ras and Torso-D^{4021}-Raf Muv phenotypes (Sieburth et al., 1998). Torso-D^{4021}-Raf contains the extracellular and transmembrane domains of a constitutively dimerizing Torso receptor tyrosine kinase fused to the kinase domain of D-Raf (Dickson et al., 1992; Baek et al., 1996). This fusion protein potentially bypasses multiple steps normally needed for Raf activation, including release of autoinhibition by the Raf N terminus, transport to and stable association with the plasma membrane, and multimerization (Morrison and Cutler, 1997). The genetic data are therefore consistent with SUR-6 functioning at any of these steps in Raf activation.

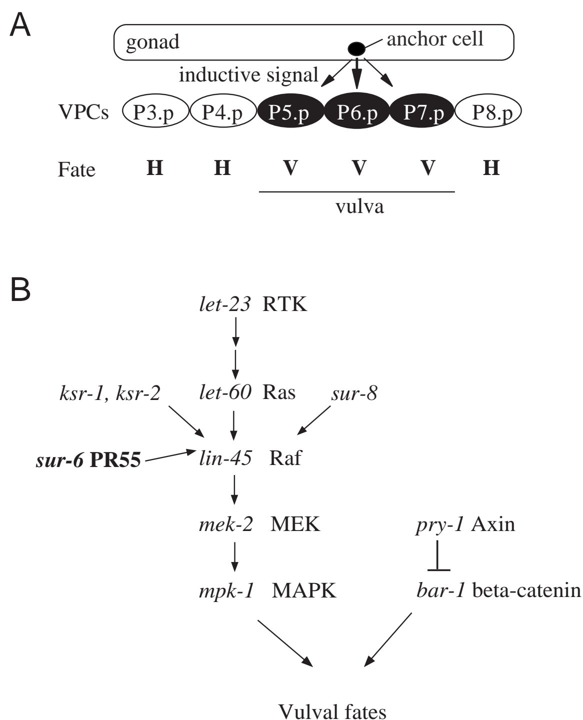

Fig. 1. *C. elegans* vulval cell fate specification. (A) Six vulval precursor cells (VPCs) are competent to adopt vulval fates in response to cell signaling events, but only three VPCs (P5.p, P6.p and P7.p) normally do so (Moghal and Sternberg, 2003). Loss-of-function mutations in the Ras pathway cause fewer than three VPCs to adopt vulval fates, whereas gain-of-function mutations in the Ras pathway cause greater than three VPCs to adopt vulval fates. (B) Ras and Wnt pathways cooperate to specify vulval cell fates (Gleason et al., 2002). The Wnt pathway behaves genetically downstream of the Ras pathway as the *pry-1* Muv phenotype is not suppressed by Ras pathway mutations (Gleason et al., 2002) but the activated MPK-1 phenotype is partly suppressed by *bar-1* mutations (Eisenmann et al., 1998). Only pathway components relevant to this paper are depicted.

The molecular identity of SUR-6 suggests a role for PP2A in modulating Raf phosphorylation. Indeed, in mammalian cells, PP2A has been proposed to activate Raf by removing inhibitory phosphates from the Raf N terminus (Abraham et al., 2000; Jaumot and Hancock, 2001; Dhillon et al., 2002; Kubicek et al., 2002). Phosphorylation of Raf-1 Ser259 by Akt and/or related serine/threonine kinases inhibits Raf activity (Zimmermann and Moelling, 1999; Rommel et al., 1999; Guan et al., 2000; Zhang et al., 2001). PP2A may dephosphorylate Raf-1 on Ser259 as one step in Raf activation. In support of this model, the PP2A catalytic subunit physically associates with Raf-1 (Abraham et al., 2000), Raf-1 Serine 259 phosphorylation increases upon treatment with the PP2A inhibitor okadaic acid (Abraham et al., 2000), and mutation of Ser259 to Ala in Raf-1 increases Raf kinase activity above basal levels (Michaud et al., 1995; Rommel et al., 1996; Clark et al., 1997). B-Raf and *C. elegans* LIN-45 RAF may be regulated in a similar manner to Raf-1 because each has multiple consensus Akt phosphorylation sites and mutation of these sites elevates their activities (Chong et al., 2001). Although the above experiments did not address which B regulatory subunit complexes with PP2A to target Raf, the data are consistent with a model in which SUR-6/PR55 and PP2A remove inhibitory phosphates from LIN-45 RAF. This model predicts that the PP2A catalytic subunit should also promote Ras signaling in *C. elegans*, and that mutating the candidate target sites on LIN-45 RAF should eliminate the requirement for SUR-6.

We analyze the effects of null mutations in *sur-6* PR55/B and *let-92* PP2A-C and provide support for the model that SUR-6 and PP2A cooperate to promote Raf activity. However, we find that mutating both consensus Akt phosphorylation sites in LIN-45 RAF does not eliminate the requirement for SUR-6. Therefore, SUR-6/PP2A does not act solely by dephosphorylating those inhibitory sites. We also provide genetic evidence that KSR activity is intact in *sur-6* mutants, and that the kinase PAR-1 functions antagonistically to SUR-6 and KSR-1 during Ras-mediated vulval induction.

Materials and methods

Genetics

General methods for growing and handling of worms were performed as described (Brenner, 1974). Worms were grown at 20°C unless stated otherwise. The wild-type strain was Bristol N2. Genes, alleles and balancers used are described (Riddle et al., 1997) unless otherwise indicated.

LGI: *unc-13(e51)*, *sur-6(ku123)* and *sur-6(cs24)* (Sieburth et al., 1999); *mek-2(h294)*, *mek-2(ku114)*, *pry-1(mu38)*, *ksr-2(dx27)* (Ohmachi et al., 2002) and *hT2[qIs48]* (Wang and Kimble, 2001).

LGIII: *mpk-1(ku1)* and *unc-119(ed3)*.

LGIV: *let-60(n1046gf)*, *let-60(n2031dn)*, *let-60 (sy100dn)*, *lin-45(ku112)* (Rocheleau et al., 2002), *lin-45(oz166)* and *lin-45(dx19)* (Hsu et al., 2002); *sur-8(ku167)* (Sieburth et al., 1998), *let-92(s504)*, *let-92(s677)*, *unc-22(s7)*, *unc-22(e66)*, *dpy-20(e1282)*, *dpy-20(e1362)*, *him-8(e1489)*, *lip-1(zh15)* (Berset et al., 2001), *nT1[qIs5]*.

LGV: *him-5 (e1490)*, *let-341(cs41)* (Rocheleau et al., 2002), *par-1(b274)*, *par-1(zu310ts)*, *rol-4(sc8)*, *unc-76(e911)*, *gaIs36* (Lackner and Kim, 1998).

LGX: *lon-2(e678)*, *ksr-1(n2526)* and *sem-5(n1779)*.

Isolation of the *sur-6(sv30)* deletion allele

A deletion library of N2 worms mutagenized with ethyl methane sulfonate (Jansen et al., 1997) was screened for deletions in the *sur-6* gene. Pooled genomic samples representing a total of 400,000 haploid genomes were used as templates in PCR reactions with two primers with the sequences 5′-CGG AGG ACA GCT GAT AAG TAA GAG GTT C-3′ and 5′-GAT GTA GAG ATT GTT AGT GGC AGC AAG AG-3′. A small amount of each reaction was used as template for a second round of PCR with the primers 5′-GAA GTT CTT CTC TGC GTG ATC GCA TAC-3′ and 5′-GAA GTT GAT CAG ATG AAA GAT CCT CTT CG-3′. The pool of worms containing the *sur-6* deletion was thawed and used to establish cultures from individual worms, from which individual heterozygous animals were identified. Sequence analysis showed that the *sv30* deletion removes 1.8 kb of the *sur-6*-coding region; it extends from exon 2 to intron 8, eliminating at least five out of the seven WD40 repeats in the SUR-6 protein. PCR experiments confirmed that a wild-type copy of the *sur-6* gene was not present elsewhere in the genome.

The *sv30* strain was outcrossed by crossing six times with wild type and by selecting for recombinants on LGI. During this procedure, a second mutation that increased the penetrance of the Vul phenotype of the strain was identified and genetically removed.

Phenotypic characterizations

Vulval development was scored in early to mid fourth larval stage (L4) animals using differential interference contrast (DIC) microscopy. Animals with fewer than 22 vulval descendants (with losses in increments of three or four cells) and greater than six non-vulval descendants were scored as vulvaless (Vul). Animals with more than 22 vulval descendants (with gains in increments of three or four cells) and fewer than six non-vulval descendants were scored as multivulva (Muv). To calculate the number of induced vulval precursor cells (VPCs) each normal lineage was given a value of 1.0 and each partial lineage was given a value of 0.5, so that wild-type animals have a value of 3.0, Vul animals have a value less than 3.0 and Muv animals had a value greater than 3.0.

Embryonic lethality was assessed by allowing hermaphrodites to lay eggs for 12-18 hours and then counting unhatched eggs 24 hours later.

Soaking RNAi

Embryos were allowed to develop on plates until most of them were at a point just before vulval development starts. The larvae were washed and ~200 larvae were mixed in an Eppendorf tube with 1 mg/ml of the appropriate dsRNA and OP50 bacteria at OD_{595} of 1.0 in a 40 μl volume followed by incubation at 20°C with gentle rotation for 24 hours. The larvae were pipetted onto seeded plates and examined for vulval development by differential interference contrast microscopy.

Immunostaining

Embryo immunostaining was performed by the freeze/crack method followed by methanol/acetone fixation (Miller and Shakes, 1995). Fixed embryos were incubated at 37°C for 1 hour with 1:100 dilution of YL1/2 rat anti-α-tubulin (Accurate Chemical & Scientific). Samples were washed twice with PBS+2% Tween-20 and incubated at 37°C for 30 minutes with 1:100 dilution of Cy3 conjugated donkey-anti rat IgG (Jackson Immuno Research) and washed three times as before. DAPI (4′,6-diamidino-2-phenylindole) was added to the penultimate wash at 0.5 mg/ml. Excess liquid was wiped off and a coverslip containing 5 μl of mowiol mounting medium was placed over the slide.

Western blotting

Worm lysates from 25-100 L4 animals were separated on 7.5% SDS-PAGE gels and transferred onto Hybond nitrocellulose (Amersham). Blots were probed with antibodies against di-phosphorylated MAPK (MAPK-YT, Sigma, 1:2500 dilution) or total MAPK (K23, Santa Cruz, 1:200 dilution) overnight at 4°C before incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research) for 1 hour at room temperature and exposure to West Pico chemiluminescent substrate (Pierce). Membranes were stripped before reprobing with the second primary antibody.

Rescue of *let-92* larval lethality

A 5.2 kb genomic fragment from the F38H4.9 locus containing 3 kb of promoter sequence and 1 kb of predicted 3′UTR was amplified from N2 genomic DNA and cloned into pBluescript II (SK+) as a *Not*I/*Kpn*I fragment to generate pGK182. Transgenic lines were generated in wild-type animals by co-injecting pGK182 at 10 ng/μl along with pTG96_2 (*sur-5::GFP*) (Yochem et al., 1998) at 30 ng/μl. Transgenic males were crossed with *let-92(s504) unc-22 (s7)/DnT1* hermaphrodites. All GFP (+) Unc-22 progeny grew up to adulthood indicating rescue of the *let-92 (s504)* larval arrest phenotype. Rescued animals were sterile indicating a role for *let-92* in the germline that could not be rescued as most *C. elegans* transgenes are transcriptionally silent in the germline (Kelly et al., 1997). The sterile phenotype of the rescued animals was similar to the sterile phenotype of wild-type larvae soaked in *let-92* dsRNA. Vulva development was normal in the rescued animals.

Site directed mutagenesis of *lin-45*

Point mutations were introduced by PCR into the *lin-45* cDNA and the mutated versions were cloned into pPD49.83 (kindly provided by A. Fire) as *Nhe*I/*Nco*I fragments to put them under control of the *hsp16-41* promoter (Stringham et al., 1992). Mutagenic primers oMS103 (5′-GAT CGG AGC TCT GCT GCT CCG AAT ATC-3′) and oMS104 (5′-GAT ATT CGG AGC AGC AGA GCT CCG ATC-3′) were used on the *lin-45* cDNA clone pRaf107a to change the codon for Ser312 to Ala in pGK167. Mutagenic primers oGK90 (5′-CGT AGT CGA GCG CCA GGC GAA CG-3′) and oGK91 (5′-CGT TCG CCT GGC GCT CGA CTA CG-3′) were then used to change Ser453 to Ala in pGK209. The *lin-45* inserts were completely sequenced to verify that the only changes were the desired ones. *lin-45(+)* cDNA was cloned into pPD49.83 to generate pGK170.

Generation of transgenic animals

A mixture of pGK167 (100 ng/μl) and pPD#MM016 (*unc-119+*) (Maduro and Pilgrim, 1995) (30 ng/μl) was injected into *unc-119(ed3)* animals. Stable extrachromosomal array lines that gave a robust Muv phenotype after heat-shock were kept. One such line was irradiated with 1800 rads of X-rays to integrate the array into the genome. One line carrying the insertion *csIs34* on the X chromosome was outcrossed four times with wild type before use in strain construction. pGK167 was injected at 20 ng/μl along with *punc-119:GFP* at 100 ng/μl to yield *csEx2*.

pGK209 was injected into N2 animals at 20 ng/μl along with pTG96_2 (*sur-5::GFP*) at 30 ng/μl and pBluescript at 50 ng/μl to yield transgenic lines *csEx52* and *csEx53*. pMS88 containing *hsp16-41::torso^{4021}-Draf* (Sieburth et al., 1998) was similarly injected to yield *csEx64*. Stable transgenes were crossed into the desired genetic backgrounds using standard genetic methods.

Results

sur-6 is essential for embryonic development but not vulval development

Although both previously described *sur-6* missense alleles are viable, RNA-mediated interference (RNAi) of *sur-6* indicated that *sur-6* is an essential gene (Sieburth et al., 1999; Piano et al., 2001). Introduction of *sur-6* double-stranded (ds) RNA into wild-type hermaphrodites (such that both maternal and zygotic *sur-6* functions were inhibited) resulted in highly penetrant embryonic lethality in the F1 generation. However, introduction of *sur-6* dsRNA into RNAi-resistant *rde-1* hermaphrodites (Tabara et al., 1999) (such that maternal *sur-6* function remained intact) had no effect on *rde-1/+* F1 progeny (R. Howard and M.V.S., unpublished). These RNAi experiments suggested that *sur-6* is required maternally but not zygotically for viability, and that *sur-6* null alleles might therefore cause maternal-effect embryonic lethality.

To further characterize *sur-6*, we generated a deletion mutation, *sv30* (Materials and methods). As predicted for a null allele, *sv30* causes maternal effect embryonic lethality (Table 1). This embryonic lethality could not be rescued by mating *sv30* hermaphrodites with wild-type males (data not shown). The deficiency *qDf8*, which removes *sur-6*, fails to complement *sv30* for the maternal effect lethal phenotype (Table 1). In addition, the *sv30/qDf8* phenotype was not more severe than that of *sv30/sv30* homozygous animals (Table 1) supporting the notion that *sv30* is a genetic null. The *sur-6(cs24)* allele complements *sv30* for the maternal effect lethal phenotype (Table 1), indicating that *cs24* does not significantly perturb the essential function of *sur-6* (see Discussion).

Table 1. The *sur-6(sv30)* null allele causes maternal effect embryonic lethality

sur-6 genotype		% embryonic	
Maternal	Zygotic	lethality (*n*)	% Vul (*n*)
*sv30/+**	*sv30/+* or *+/+**	0 (167)	0 (22)
*sv30/+**	*sv30/sv30**	0 (55)	4 (52)
sv30/sv30	*sv30/sv30*	100 (1661)	NA
sv30/cs24†	*sv30/sv30*†	≤2 (334)	18 (16)
sv30/cs24†	*sv30/cs24*†	≤2 (668)	12 (32)
cs24/cs24	*cs24/cs24*	0 (514)	2 (48)‡
+/qDf8§	*sv30/qDf8*§	ND	10 (29)
sv30/qDf8§	Multiple§	100 (335)	NA

**sur-6(sv30)/+* hermaphrodites yielded 100% viable progeny (*n*=222). Genotypes of F1 progeny were assessed by progeny testing. To score vulval phenotypes, *sv30* heterozygotes and homozygotes were recognized, respectively, as GFP (–) or GFP (+) segregants from *sur-6(sv30)/hT2[qIs48, GFP (+)]* mothers. *qIs48* is a recessive lethal insertion of *pes-10::GFP, myo-2::GFP* and *F22B7::GFP* reporters into the balancer chromosome (Wang and Kimble, 2001).

†Among the brood of *sur-6(sv30)/unc-13 sur-6(cs24)* hermaphrodites, only 2% arrested as embryos (*n*=1337). Surviving progeny of a single *sur-6(sv30)/unc-13(e51) sur-6(cs24)* mother were picked for progeny testing: 22% were Unc, 55% were heterozygotes and 22% were *sur-6(sv30)* homozygotes (*n*=81). Final *n* values were inferred from this sampling. Genotypes of animals scored for vulval phenotypes were assessed by progeny testing. Pair-wise testing by Fisher's exact test of the Vul phenotype of these strains with the Vul phenotype of *sv30/sv30* animals from *sv30/+* mothers produced *P* values greater than 0.05.

‡Sieburth et al., 1999.

§*sv30/qDf8* animals were recognized as non-Dpy, GFP (–) segregants from a cross between *qDf8/hT2[qIs48, GFP (+)]* males and *sur-6(sv30)/hT2[qIs48, GFP (+)]*; *dpy-20* hermaphrodites. The dead embryos produced by *sv30/qDf8* mothers were of three different genotypes: *sv30/sv30*, *sv30/qDf8* and *qDf8/qDf8*.

ND, not done; NA, not applicable.

We examined *sur-6(sv30)* and *sur-6(RNAi)* embryos to

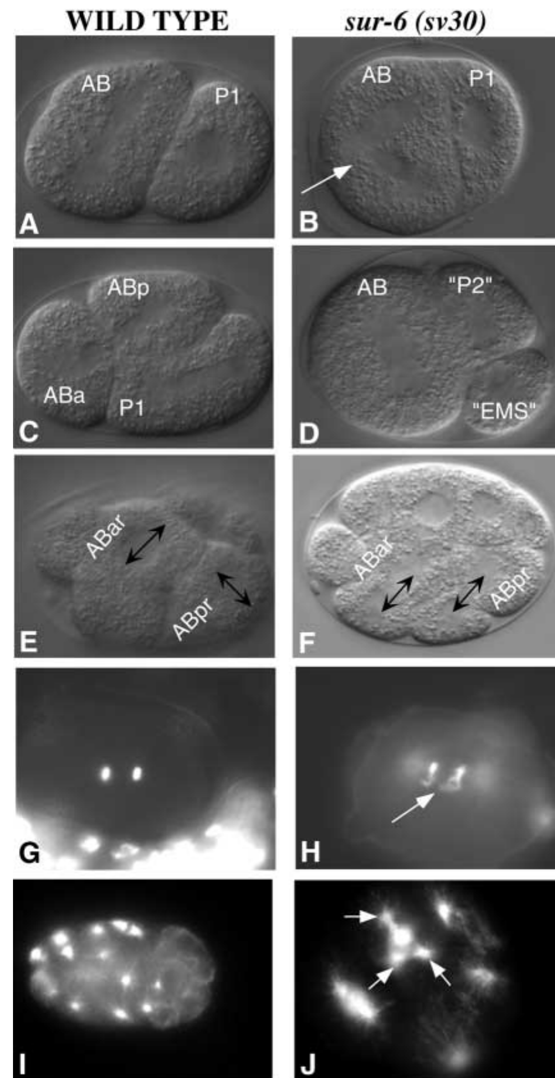

Fig. 2. Abnormal cell divisions in *sur-6(sv30)* embryos. Cell division patterns (A-F), chromosome segregations during anaphase (G,H) and spindle orientation (I,J) were examined in wild-type embryos (A,C,E,G,I) or embryos produced by *sur-6(sv30)* mothers (B,D,F,H,J). (A-F) Nomarski images. (A) Wild-type embryo showing normal anaphase spindle in the AB cell. (B) *sur-6(sv30)* embryo showing an abnormal L-shaped spindle in the AB cell. (C) Wild-type embryo in which AB has divided before P1. (D) *sur-6(sv30)* embryo in which P1 has divided before AB. (E) Wild-type embryo showing the orthogonal orientation of the ABar and ABpr spindles. Double-headed arrows indicate the orientation of the spindle. (F) *sur-6(sv30)* embryo showing parallel orientation of the ABar and ABpr spindles. A similar defect has been reported in some Wnt pathway mutants (Rocheleau et al., 1997; Thorpe et al., 1997). (G,H) DAPI staining. (G) Wild-type one-cell embryo showing a normal anaphase figure. (H) *sur-6(sv30)* one cell embryo with anaphase bridging defects (arrow). (I,J) Anti-tubulin staining. (I) Wild-type multicellular embryo showing two centrosomes per dividing cell. (J) *sur-6(sv30)* multicellular embryo showing a dividing cell with supernumerary centrosomes (arrows).

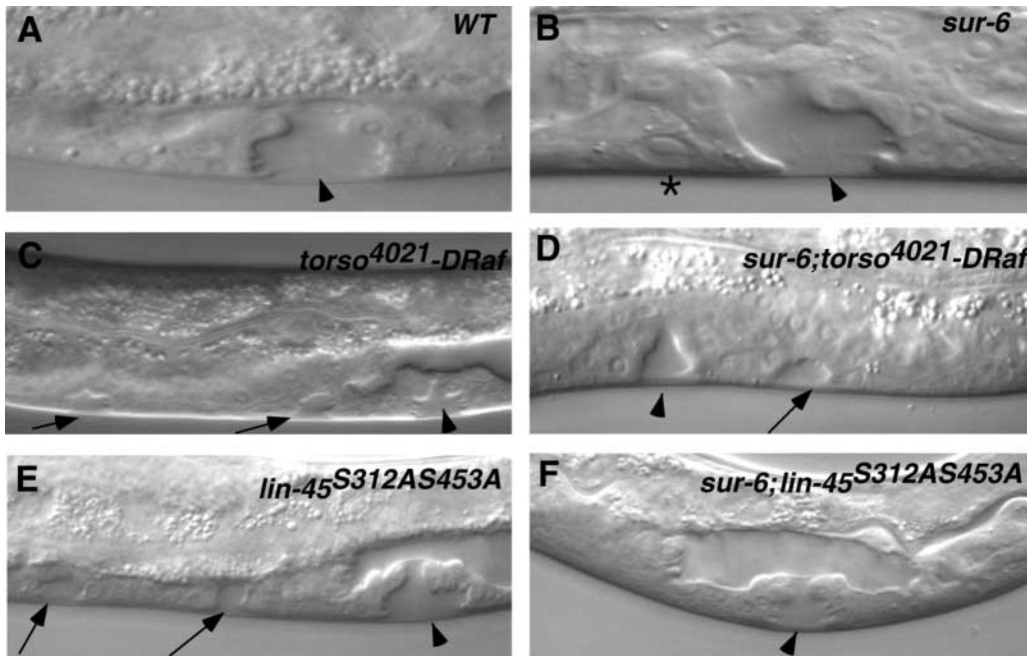

Fig. 3. *sur-6(sv30)* suppresses the *lin-45*$^{S312A\ S453A}$ Muv phenotype. Animals were observed by Nomarski optics at the L4 larval stage. Arrowheads indicate the real vulva, arrows indicate ectopic vulval invaginations (C,E,F) and asterisk in B indicates a non-vulval fate for P7.px in a partially vulvaless animal. (A) Wild type; (B) *sur-6(sv30)*; (C) *hs-torso*^{4021}*-Draf;* (D) *sur-6(sv30); hs-torso*^{4021}*-Draf.* (E) *hs-lin-45* $^{S312A\ S453A}$; (F) *sur-6(sv30); hs-lin-45*$^{S312A\ S453A}$.

determine the cause of lethality. Both types of embryos appeared similar and exhibited spindle defects and abnormal timing of the earliest cell divisions (Fig. 2). In wild-type embryos, the zygote divides asymmetrically to generate a larger anterior blastomere (AB) and a smaller posterior blastomere (P1). AB then divides symmetrically to generate ABa and ABp (Fig. 2A), and shortly thereafter P1 divides asymmetrically to generate EMS and P2 (Fig. 2C). In *sur-6(sv30)* embryos, P1 often initiated division prior to AB (Fig. 2D, 4/11 embryos), spindles appeared morphologically abnormal or collapsed during anaphase (Fig. 2B, 4/11 embryos), and chromatin bridges (Fig. 2H) and supernumerary centrosomes (Fig. 2J) were observed by DAPI and anti-tubulin staining, respectively. These defects suggest a general requirement for *sur-6* in mitotic progression during embryogenesis.

Maternally rescued *sur-6(sv30)* homozygotes do not, however, show obvious cell cycle defects. They have normal gonadal and germline morphology and are fertile, but are mildly uncoordinated (Unc) and have a weakly penetrant Vul defect that resembles the Vul defect of *sur-6(cs24)* missense mutants (Table 1, Fig. 3B). The relatively weak Vul phenotype could be explained in part by perdurance of maternally provided gene product. However, *sur-6(sv30)* homozygotes from *sur-6(sv30)/sur-6(cs24)* mothers have only marginally more severe Vul defects than *sur-6(sv30)* homozygotes from *sur-6(sv30)/+* mothers (Table 1). Therefore, it appears that *sur-6* promotes but is not absolutely essential for vulval induction.

sur-6 mutations decrease MPK-1 ERK phosphorylation

In the context of Ras pathway activity, *sur-6(sv30)* behaves similarly to the previously described *sur-6* missense alleles. The *sur-6(sv30)* Vul phenotype is strongly enhanced by hypomorphic alleles of *lin-45 raf* or the Raf regulator *sur-8*

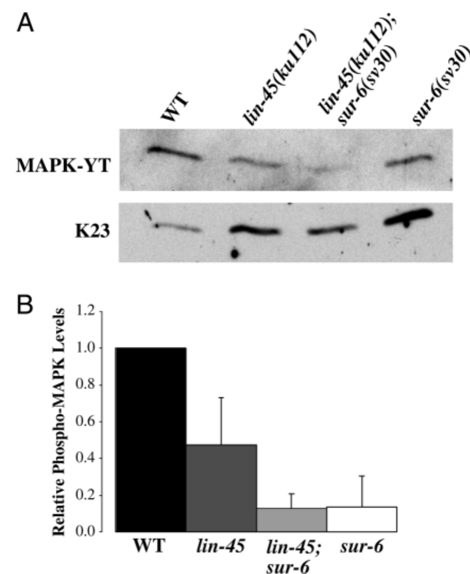

Fig. 4. Activated MAP-kinase levels in wild-type and *sur-6(sv30)* mutant backgrounds. Western blots were performed using L4 animals. Membranes were probed with MAPK-YT antibody (Sigma) specific for dually phosphorylated MAP kinase, then stripped and reprobed with K23 antibody (Santa Cruz Biotechnology) which detects total MAP kinase for use as a loading control. Experiments were carried out in triplicate. (A) One representative western blot is shown. The *mpk-1/*map-kinase gene produces a 45 kDa soma–specific isoform in larvae (M. H. Lee and T. Schedl, personal communication). MAPK-YT reactive bands were absent in *mek-2(h294lf)* animals (Ohmachi et al., 2002). *lin-45(ku112)* animals were included as a positive control for reduced MPK-1 ERK phosphorylation. (B) Quantitation of three independent western blots was carried out using a BioRad GS670 imaging densitometer. Bands visualized by the K23 antibody were used as the loading control for the normalization of intensities of the bands visualized by the use of MAPK-YT.

(Table 2A). *sur-6(sv30)* also dominantly suppresses the Muv phenotype caused by an activated allele of *let-60 ras*, but does not suppress the Muv phenotypes caused by activated *torso*^{4021}*-D-raf* or by activated forms of MEK and ERK (Table 2B, Fig. 3C,D). We also found that *sur-6(sv30)* fails to suppress the Muv phenotype caused by loss of *pry-1* Axin, a negative regulator of *bar-1* β-catenin (Gleason et al., 2002; Korswagen et al., 2002), confirming that *sur-6* specifically affects Ras signaling and not Wnt signaling (Table 2B). Finally, western blot analyses using a monoclonal antibody specific for the dually phosphorylated active form of ERK revealed a fivefold decrease in MPK-1 ERK phosphorylation in *sur-6(sv30)* mutants (Fig. 4). These data support the model that *sur-6* promotes Ras signaling and acts upstream of Raf.

Table 2. Epistasis analysis with *sur-6(sv30)*

A Epistasis in sensitized mutant backgrounds

Row	Genotype*	% Vul†	Average number of VPCs induced	*n*
(1)	*sur-6*	4	2.9	52
(2)	*sur-6/+; lin-45(ku112)*	0	3.0	21
(3)	*sur-6; lin-45(ku112)*	96** (1,2)	0.2	20
(4)	*sur-6/+; sur-8*	0	3.0	21
(5)	*sur-6; sur-8*	100** (1,4)	0.3	22
(6)	*sur-6/+; ksr-1*	0	3.0	20
(7)	*sur-6; ksr-1*	10	2.9	21
(8)	*ksr-2*	0	3.0	14‡
(9)	*sur-6 ksr-2*	5	2.9	101

B Epistasis with multivulva strains

Row	Genotype§	% Muv†	Average number of VPCs induced	*n*
(10)	*let-60(gf)*	75	3.7	20
(11)	*sur-6/+; let-60(gf)*	14	3.1	21
(12)	*sur-6; let-60(gf)*	10†† (10)	3.1	20
(13)	*hs-torso*^{4021}*-Draf*	23	3.4	48
(15)	*sur-6/+; hs-torso*^{4021}*-Draf*	19	3.1	47
(16)	*sur-6; hs-torso*^{4021}*-Draf*	14	3.1	15
(17)	*E1F-Dmek, hs-mpk-1*	53	3.5	38
(18)	*sur-6/+;E1F-Dmek, hs-mpk-1*	67	3.6	21
(19)	*sur-6; E1F-Dmek, hs-mpk-1*	63	3.8	38
(20)	*pry-1 (25°C)*	15	3.1	20
(21)	*sur-6; pry-1 (25°C)*	20	3.2	20

C Epistasis with *lin-45(gf)* transgenes

Row	Genotype¶	% Vul†	% Muv†	Average number of VPCs induced	*n*
(22)	*hs-lin-45*^{S312A}	0	59	3.6	111
(23)	*sur-6/+; hs-lin-45*^{S312A}	0	35** (22)	3.3	155
(24)	*sur-6; hs-lin-45*^{S312A}	0	4** (22)	3.02	45
(25)	*hs-lin-45*$^{S312A\ S453A}$	0	69	3.7	58
(26)	*sur-6/+; hs-lin-45*$^{S312A\ S453A}$	0	42** (25)	3.4	43
(27)	*sur-6; hs-lin-45*$^{S312A\ S453A}$	0	0** (25)	3.0	34
(28)	*lin-45(dx19)*	100	0	0.1	11
(29)	*lin-45(dx19); hs-lin-45*$^{S312A\ S453A}$	11	68** (28)	3.5	19
(30)	*hs-lin-45*^{S312A}	0	31	3.6	36
(31)	*ksr-1; hs-lin-45*^{S312A}	19	0†† (30)	2.5	21
(32)	*hs-lin-45*^{S312AS453A}	0	68	4.0	35
(33)	*ksr-1; hs-lin-45*^{S312AS453A}	0	16** (32)	3.2	18

*Alleles used were sur-6(sv30), lin-45(ku112), sur-8(ku167), ksr-1(n2526) and ksr-2(dx27).
sur-6 and *ksr-2* homozygotes were recognized as GFP (–) segregants from *hT2[qIs48GFP+]* balanced strains*. ksr-1*was linked to *lon-2(e678).*

†% Vul (vulvaless) and % Muv (multivulva) were scored as described in the Materials and methods. Statistical analysis was performed using Fisher's exact test. ***P*≤0.001, ††*P*≤0.02. The numbers in brackets indicate the row with which the data were compared. Where not indicated, *P*≥0.2.

‡Ohmachi et al., 2002.

§Alleles used were *sur-6(sv30), let-60(n1046gf)* and *pry-1(mu38)*. Extrachromosomal array *csEx64* was used as a source of *hs-torso*^{4021}*-Draf*. *csEx64* bearing larvae were heat shocked at 38°C for 45 minutes, 47 hours after egg-lay. The integrated transgene *gaIs36(E1F-D-mek, hs-mpk-1)* was linked to *him-5(e1490)*. *gaIs36*-bearing animals were transferred from 15°C to 25°C as embryos.

¶Alleles used were *sur-6(sv30)*, *lin-45(dx19)* and *ksr-1(n2526)*. *sur-6(sv30)* experiments with *lin-45*^{S312A} (rows 22-24) were carried out using the integrated transgene *csIs34*. *sur-6(sv30)* and *lin-45(dx19)* experiments with *lin-45*$^{S312A\ S453A}$ (rows 25-29) were performed using *csEx52*. *ksr-1* experiments with *lin-45*^{S312A} (rows 30 and 31) were carried out with *csEx2* and those with *lin-45*$^{S312A\ S453A}$ (rows 32 and 33) were carried out with *csEx53*. Animals transgenic for *hsp-lin-45*$^{+}$ did not produce a Muv phenotype upon heat shock in wild-type and *sur-6* backgrounds. *csIs34*- and *csEx2*-bearing larvae were heat-shocked at 37°C for 30 minutes as mid to late second larval stage (L2) animals (42 hours post-egg lay). *csEx52*- and *csEx53*-bearing larvae were heat-shocked at 34°C for 35 minutes as mid to late L2 (43 hours post egg-lay). We do not yet know the reason for the partial Vul phenotype in *ksr-1; csEx2* animals.

let-92/PP2A-C cooperates with *sur-6*/PR55 during embryonic development

We next wanted to test the relationship between SUR-6 and the PP2A catalytic core. F38H4.9 encodes the single catalytic subunit of PP2A (PP2A-C) in *C. elegans*. Introduction of F38H4.9 dsRNA into wild-type hermaphrodites resulted in embryonic lethality in the F1 generation (Sieburth et al., 1999; Kamath et al., 2003), whereas introduction of F38H4.9 dsRNA into *rde-1* hermaphrodites resulted in early larval lethality in the *rde-1/+* F1 progeny (R. Howard and M.V.S., unpublished). These RNAi experiments suggested that PP2A-C is required both maternally and zygotically for viability, and that PP2A-C mutations might cause larval lethality. The cosmid B0033 and a plasmid containing the F38H4.9 gene can rescue the larval lethality of *let-92* mutants (see Materials and methods), suggesting that PP2A-C corresponds to the genetic locus *let-92* (Rogalski and Baillie, 1985). This assignment has been independently established (Ogura et al., 2003). We henceforth refer to F38H4.9/PP2A-C as *let-92*.

let-92(RNAi) embryos showed catastrophic failures in cytokinesis during the earliest cell divisions (data not shown). These defects are more severe than those seen in *sur-6* mutant embryos, consistent with studies in yeast and mammals that suggest the catalytic subunit acts in concert with multiple regulatory subunits to participate in distinct cellular and developmental events (Janssens and Goris, 2001). Although the *sur-6* and *let-92* embryonic arrest phenotypes are distinct, a *let-92* loss-of-function mutation shows strong dominant synthetic lethal interactions with both viable *sur-6* missense alleles (Table 3), suggesting that *sur-6* and *let-92* do function together during embryogenesis.

Table 3. *sur-6* missense alleles and *let-92/+* display synthetic embryonic lethality

Row	Maternal genotype*	% F1 lethal†	*n*
(1)	*+; let-92/+*	25	684
(2)	*sur-6(cs24);+*	5	126
(3)	*sur-6(cs24); let-92/+*	93** (1)	306
(4)	*sur-6(ku123);+*	<1	114
(5)	*sur-6(ku123); let-92/+*	88** (1)	342

*Chromosome I was marked with *unc-13(e51)*. *let-92(s504)* chromosomes were marked with *unc-22(s7)* and balanced with *dpy-20(e1282)*.

†Expected lethality in brood of *let-92/+* mother is 25%, and the lethal progeny die as larvae. In contrast, most lethal progeny from *sur-6; let-92/+* mothers die as embryos. Statistical analysis was performed using Fisher's exact test. $^{**}P\leq0.001$. The numbers in brackets indicate the row with which the data were compared.

let-92/PP2A-C is a positive regulator of vulval development

The different regulatory subunits of PP2A can either promote or inhibit the catalytic activity of PP2A towards different substrates, and both cooperative and antagonistic relationships between different PP2A subunits have been documented in *S. cerevisiae* and *Drosophila* (van Wyl et al., 1992; Maixner et al., 1998; Janssens and Goris, 2001). We sought to determine if *sur-6* promotes or inhibits PP2A activity towards a Ras pathway substrate by examining the effect of reducing *let-92* activity. If *sur-6* promotes PP2A activity then reducing *let-92* should cause a Vul phenotype similar to that caused by *sur-6(sv30)*, whereas if *sur-6* inhibits PP2A activity then reducing *let-92* should have the opposite effect.

Our previous experiments suggested a positive role for *let-92* because, like *sur-6(RNAi)*, *let-92(RNAi)* could partially suppress the *let-60(gf)* Muv phenotype (Sieburth et al., 1999). We also found that *let-92(RNAi)* and *sur-6(RNAi)* caused similar weak synthetic Vul phenotypes in *sur-8* mutant larvae (Table 4), further supporting a positive role for *let-92*.

Homozygous *let-92* mutants die as very young larvae (Rogalski and Baillie, 1985), before vulval development starts, preventing us from directly examining vulval development in these strains. Instead, we tested *let-92* alleles for dominant genetic interactions with other Ras pathway components. *let-92/+* did not modify the *let-60(gf)/+* Muv phenotype (Table 4). However, *let-92/+* strongly enhanced the penetrance and expressivity of the Vul defects caused by two different dominant-negative alleles of *let-60 ras* (Table 4). This effect of *let-92* was similar to that of a null allele of *lin-45 raf* (Table

Table 4. *let-92* is a positive regulator of vulval fate specification

Row	Genotype*	% Vul†	% Muv†	Number of VPCs induced	*n*
(1)	*sur-6(RNAi)*	0	0	3.0	12
(2)	*let-92(RNAi)*	0	0	3.0	86
(3)	*sur-8*	0	0	3.0	62
(4)	*sur-8; sur-6(RNAi)*	10** (1,3)	0	2.9	20
(5)	*sur-8; let-92(RNAi)*	10** (2,3)	0	2.9	91
(6)	*let-92(s504)/+*	0	0	3.0	22
(7)	*let-92(s677)/+*	0	0	3.0	20
(8)	*let-60(sy130gf)/+*	0	6	ND	72
(9)	*let-60(sy130gf)/let-92(s504)*	0	7	ND	100
(10)	*let-60(n2031dn)/+*	53	0	2.2	34
(11)	*let-60(n2031dn)/let-92(s504)*	93** (10)	0	0.8	59
(12)	*let-60(n2031dn)/let-92(s677)*	84** (10)	0	1.3	25
(13)	*let-60(n2031dn)/lin-45(oz166)*	84§ (10)	0	1.1	19
(14)	*let-60(sy100dn)/+*	71	0	1.9	24
(15)	*let-60(sy100dn)/let-92(s504)*	100§ (14)	0	0.4	24

**let-92(s504)* and *let-92(s677)* were marked with *unc-22(s7)* and balanced in trans to *dpy-20 (e1282)*. *let-60(sy130gf)* and *let-60(sy100dn)* were marked with *dpy-20(e1282)*. *let-60 (n2031dn)* and *let-60(sy100dn)* were balanced in trans to *unc-22(s7)*.

†% Vul (vulvaless) and % Muv (multivulva) were scored as described in the Materials and methods. Statistical analysis was performed using Fisher's exact test. $^{**}P\leq0.001$, $^{§}P\leq0.02$. The numbers in brackets indicate the row with which the data were compared. ND, not determined.

4), consistent with *let-92* mutations causing a reduction in Raf activity. *let-92/+* did not affect the vulval defects of partial loss-of-function alleles of other Ras pathway genes such as *sem-5/*Grb2, *let-341*/Sos, *mek-2*/MEK, *mpk-1*/ERK or *lip-1*/ MKP (data not shown, see Materials and methods for alleles used). Therefore, sensitivity to reduced *let-92* dosage may be specific for the Ras/Raf level of the signal transduction pathway.

Based on these experiments, we conclude that *let-92*, like *sur-6*, plays a positive role in vulval fate induction. We find no evidence for a negative role. Thus, *sur-6* most probably promotes PP2A activity toward a Ras pathway substrate. If so, SUR-6/PP2A either removes inhibitory phosphates from a positively acting substrate or removes stimulatory phosphates from a negatively acting substrate.

sur-6 functions independently of inhibitory phosphorylation sites on LIN-45 RAF

We tested whether SUR-6/PP2A acts by removing inhibitory phosphates from LIN-45 RAF. Mammalian Raf-1 has a single consensus Akt phosphorylation site (Serine 259) that is subject to inhibitory phosphorylation and that PP2A has been proposed to dephosphorylate (Chong et al., 2003). *C. elegans* LIN-45 RAF has two consensus Akt phosphorylation sites (Ser312 and Ser453) that appear to function analogously to the single Raf-1 Ser259 site (Chong et al., 2001). We mutated both serines to alanine and expressed the mutant LIN-45 proteins under the control of the heat shock promoter *hsp16-41* (Stringham et al., 1992). When overexpressed in this manner, LIN-45^{+} had no apparent effect (data not shown). However, LIN-45^{S312A} caused a moderate Muv phenotype and LIN-45$^{S312A\ S453A}$ caused a somewhat stronger Muv phenotype (Table 2C, Fig. 3E). These results are similar to those reported previously (Chong et al., 2001), except that those authors saw a Muv phenotype with LIN-45$^{S312A\ S453A}$ but not LIN-45^{S312A}, possibly because of lower expression levels. Taken together, our results are consistent with the model that both Ser312 and Ser453 in LIN-45 RAF are sites of inhibitory phosphorylation.

If Ser312 and Ser453 in LIN-45 RAF are the relevant targets of SUR-6/PP2A during vulval development, then mutation of those serines to alanine should eliminate the need for *sur-6*. However, the Muv phenotypes of LIN-45^{S312A} and LIN-45$^{S312A\ S453A}$ still required *sur-6* (Table 2C, Fig. 4E,F). This requirement for *sur-6* cannot be explained by an effect on endogenous LIN-45^{+} as LIN-45$^{S312A\ S453A}$ produced a potent Muv phenotype even in a strong loss-of-function *lin-45(dx19lf)* mutant background (Table 2C). Because removing both inhibitory sites did not eliminate the requirement for *sur-6*, SUR-6 must promote LIN-45 RAF activity via a mechanism distinct from dephosphorylating those sites. SUR-6/PP2A may regulate LIN-45 RAF through as yet unidentified phosphorylation sites, or it may regulate LIN-45 RAF indirectly by targeting other Raf regulatory proteins.

sur-6 functions independently of *ksr-1* or *ksr-2*

The putative scaffold protein KSR is a positive Raf regulator whose function can be inhibited by phosphorylation (Muller et al., 2001), making it another candidate PP2A substrate. Furthermore, KSR and SUR-6 appear to act at a similar step of Raf activation, as *ksr-1* also suppresses the Muv phenotype caused by LIN-45$^{S312A\ S453A}$ but not Torso^{4021}-Draf (Table 2B,C) (Sieburth et al., 1999). *C. elegans* has two *ksr* genes, *ksr-1* and *ksr-2*, that are redundantly required for viability and vulval development (Ohmachi et al., 2002). Although *ksr-1* and *ksr-2* single mutants are viable and have normal vulvae, the mutants are very sensitive to further reductions in KSR activity (Ohmachi et al., 2002). Therefore, if *sur-6* mutations reduce KSR activity, we would expect to see a strong genetic interaction between a *sur-6* null mutation and *ksr-1* or *ksr-2*. Contrary to this prediction, *ksr-1* and *ksr-2* mutations each failed to enhance *sur-6(sv30)* Vul defects (Table 2A). These results suggests that KSR activity is relatively intact in *sur-6* mutants. Therefore, we do not favor the model that KSR is a key substrate of SUR-6/PP2A.

Table 5. *par-1* acts antagonistically to *sur-6* and *ksr-1*

Row	Genotype*	%Muv†	Number for VPCs induced	*n*
(1)	*par-1(b274)*	0	3.0	12
(2)	*par-1(RNAi)*	0	3.0	52
(3)	*par-1(b274)/par-1(zu310ts)*	9** (1)	3.1	22
(4)	*let-60(gf)*	100	4.7	13
(5)	*par-1(b274); let-60(gf)*	100	4.6	14
(6)	*sur-6; let-60(gf)*	0	3.0	22
(7)	*sur-6; par-1(b274); let-60(gf)*	65** (6)	3.5	20
(8)	*let-60(gf); ksr-1*	4	3.03	43
(9)	*let-60(gf); ksr-1; par-1(RNAi)*	38** (8)	3.3	47
(10)	*lin-45 let-60(gf)*	0	3.0	30
(11)	*par-1(b274); lin-45 let-60(gf)*	0	3.0	17

*Alleles used were *sur-6(ku123)*, *ksr-1(n2526)*, *let-60(n1046gf)* and *lin-45(ku112)*. The maternal-effect lethal allele *par-1(b274)* was linked to *rol-4(sc8)*. *rol-4(sc8)* alone had no effect on the *sur-6; let-60(gf)* phenotype. The temperature-sensitive allele *par-1(zu310)* was linked to *unc-76(e911)*. For *par-1(RNAi)*, vulval development was scored in the progeny laid between 3 hours and 16 hours after dsRNA injection; all progeny laid after this time arrested during embryogenesis as expected based on the *par-1* loss-of-function phenotype (Guo and Kemphues, 1995).

†Statistical analysis was performed using Fisher's exact test. ***P*≤0.001. The numbers in brackets indicate the row with which the data were compared.

The PAR-1 kinase acts antagonistically to SUR-6 and KSR during vulval development

The serine/threonine kinase C-TAK1/PAR-1 has been identified biochemically as an inhibitor of mammalian KSR (Muller et al., 2001). Most *C. elegans par-1* mutants have wild-type vulval fate specification (Hurd and Kemphues, 2003). However, we find that *par-1(b274lf)/par-1(zu310ts)* trans-heterozygotes (in which both maternal and zygotic *par-1* activities are reduced) are weakly Muv (Table 5). Additionally we find that reducing *par-1* function strongly reverts the suppressed (non-Muv) phenotype of *sur-6(ku123);let-60(n1046gf)* and *let-60(n1046gf);ksr-1(n2526)* double mutants (Table 5). By contrast, reducing *par-1* function does not revert the suppressed (non-Muv) phenotype of *lin-45(ku112) let-60(n1046gf)*. Therefore *par-1* has an inhibitory role in vulval development, probably acts upstream or parallel to *lin-45 raf*, and functions antagonistically to *sur-6/*PP2A and *ksr-1*.

Discussion

Our results suggest that SUR-6, the PR55/B regulatory subunit of PP2A, and LET-92, the catalytic subunit of PP2A, cooperate to positively regulate mitotic progression and Ras signaling in *C. elegans*. Although we have been unable to use epistasis

analysis to place LET-92/PP2A at a particular step of the Ras pathway, the simplest model is that SUR-6/PR55 and LET-92/PP2A act at the same step. Epistasis analysis using a *sur-6* null allele supports the model that SUR-6 acts upstream of LIN-45 RAF, but is inconsistent with the model that SUR-6 and PP2A act solely by dephosphorylating inhibitory Akt sites on LIN-45 RAF. Instead, SUR-6 and PP2A may regulate Raf indirectly by influencing another Raf regulatory protein.

Positive versus negative roles for PR55/PP2A in Ras signaling

In other systems, PR55/B and PP2A have been found to have both positive and negative effects on Ras signaling. For example, in *Drosophila* a positive role for PR55/PP2A is supported by findings that mutations in *tws*/PR55 suppress the lethality caused by activated sevenless receptor and activated Ras (Maixner et al., 1998), and mutations in the PP2A catalytic subunit enhance photoreceptor defects caused by a hypomorphic Draf allele (Wassarman et al., 1996). However, a negative role for PR55/PP2A is supported by findings that RNAi against *tws*/PR55 elevates the level of phospho-ERK in cultured S2 cells (Silverstein et al., 2002), and mutations in the PP2A catalytic subunit enhance photoreceptor defects caused by activated Ras (Wassarman et al., 1996). Thus, in *Drosophila* the role of PR55/PP2A appears complex, and PP2A may act on multiple substrates within the Ras pathway. Similarly, in mammalian cells PP2A has been suggested to positively regulate Ras signaling by removing inhibitory phosphates from Raf (Abraham et al., 2000; Jaumot and Hancock, 2001; Dhillon et al., 2002; Kubicek et al., 2002) and to negatively regulate Ras signaling by removing activating phosphates from MEK or ERK (Alessi et al., 1995). By contrast, we find no evidence for a negative role of SUR-6/PR55 or LET-92/PP2A in *C. elegans*, despite having tested *sur-6* and *let-92* mutations in numerous genetic backgrounds. Therefore either PP2A lacks a negative role in *C. elegans*, or its negative role is masked by its stronger positive role.

SUR-6/PP2A as a positive regulator of LIN-45 RAF activity

Prior studies of SUR-6 and its genetic placement between (or in parallel to) Ras and Raf relied on partial loss-of-function *sur-6* alleles (Sieburth et al., 1999). Our characterization of a *sur-6* null mutation, *sv30*, is consistent with the prior studies but clarifies several previously unresolved points. First, our data suggest that SUR-6 promotes Ras signaling but is not absolutely essential for Ras signaling under normal circumstances. Therefore, SUR-6/PP2A may dephosphorylate a site that has modest effects on substrate activity, or SUR-6/PP2A function may be partly redundant with that of another phosphatase complex. Second, our finding that *sur-6(sv30)* reduces the levels of activated MPK-1 ERK in vivo argues that SUR-6 acts upstream of rather than in parallel to MEK and ERK. SUR-6 could still act either upstream or in parallel to LIN-45 RAF. Finally, our data dispute two of the prevailing models for SUR-6 function.

Like mammalian Raf proteins, LIN-45 RAF appears to be inhibited by phosphorylation on sites that match the consensus sequence for the Akt kinase (Chong et al., 2001). To date we have not seen effects of *akt-1* or *akt-2* RNAi on Ras signaling (G.K. and M.V.S., unpublished), so it is unclear whether Akt itself or some other kinase(s) normally phosphorylates these LIN-45 RAF inhibitory sites. Although a simple and attractive model was that SUR-6 and PP2A dephosphorylate these LIN-45 RAF inhibitory sites, our data are inconsistent with that being their sole mechanism of action. We found that *sur-6(sv30)* suppresses the Muv phenotype caused by LIN-$45^{S312A\ S453A}$, which lacks both presumptive inhibitory Akt phosphorylation sites, indicating that SUR-6 must promote LIN-45 RAF activity independently of those sites. One possibility is that SUR-6/PR55 and PP2A dephosphorylate LIN-45 RAF on other inhibitory sites; however, no such sites have been identified as yet. An alternative possibility is that SUR-6 and PP2A indirectly influence LIN-45 RAF by dephosphorylating some other Raf regulatory protein(s).

A second proposed model was that SUR-6 and PP2A regulate the scaffold protein KSR (Sieburth et al., 1999; Ory et al., 2003). KSR may regulate Raf at a similar step as SUR-6, as *ksr-1* mutations also suppress the Muv phenotype caused by LIN-$45^{S312A\ S453A}$ but not $Torso^{4021}$-Draf. However, as discussed above, the failure of *sur-6* mutations to genetically interact with *ksr-1* or *ksr-2* suggests that KSR activity is relatively intact in *sur-6* mutants. Therefore, KSR is unlikely to be the sole SUR-6/PP2A substrate. It remains possible that SUR-6/PP2A has multiple substrates, and that LIN-45 RAF and/or KSR are among these, but our data argue that another important substrate(s) remains to be identified.

The kinase PAR-1 acts in opposition to PP2A

Murine C-TAK/PAR-1 has been suggested to phosphorylate KSR to modulate KSR localization (Muller et al., 2001). The relevant phosphorylation site is not conserved in *C. elegans* KSR-1 or KSR-2, although related sites are present elsewhere. PAR-1 can also phosphorylate Raf as well as other substrates (Benton et al., 2002) and therefore could have broader roles in Ras signaling. *C. elegans par-1* has been previously found to play a role in vulval morphogenesis (Hurd and Kemphues, 2003). We found that *par-1* also plays an inhibitory role in vulval fate specification. This inhibitory role of *par-1* is partly masked by perdurance of maternally provided gene product, but could be seen in animals in which both maternal and zygotic *par-1* contributions were diminished, as well as in some sensitized genetic backgrounds. We found that zygotic removal of *par-1* reverts the suppressor of *ras (gf)* phenotypes of *sur-6* and *ksr-1* but not *lin-45 raf*, consistent with models where PAR-1 acts on KSR, LIN-45 RAF or both to inhibit vulval development. Our results also raise the possibility that SUR-6/PP2A inhibits PAR-1 to indirectly affect LIN-45 RAF activity.

Genetically separable functions for SUR-6/PR55 in Ras signaling and mitotic progression

The *sur-6* maternal effect lethal phenotype reveals that in addition to Ras signaling, *sur-6* is required for mitotic progression. *sur-6(sv30)* and *sur-6(RNAi)* embryos display a variety of mitotic defects such as ectopic and aberrant cytokinesis, the collapse and re-elaboration of well-extended anaphase spindles, abnormally shaped spindles and chromatin bridges during anaphase. Similar mitotic defects have been observed in *Drosophila tws*/PR55 mutants (Gomes et al., 1993; Mayer-Jaekel et al., 1993). Premature sister chromatid separation and cytokinesis defects have also been observed in

S. cerevisiae cdc55/PR55 mutants (Minshull et al., 1996; Wang and Burke, 1997). Thus, the mitotic role of PR55 appears to be evolutionarily conserved. The early *C. elegans* embryo is a particularly tractable system for further study of this poorly understood mitotic role of PR55.

Interestingly, the original two missense alleles of *sur-6*, *ku123* and *cs24*, behave similarly to the *sur-6* null allele *sv30* with respect to Ras signaling but do not cause mitotic defects or embryonic lethality even when placed in trans to *sur-6(sv30)* or a deficiency of the *sur-6* locus as shown here and previously (Sieburth et al., 1999). Thus, the functions of *sur-6* in Ras signaling and mitotic progression are genetically separable. The *cs24* allele causes an E to K change at an absolutely conserved site of the third WD repeat of SUR-6/PR55 (Sieburth et al., 1999). Equivalent mutations in the human PR55 protein have been shown to severely compromise binding of PR55 to the A subunit of PP2A, preventing PR55 association with the catalytic core (Strack et al., 2002). Thus, *cs24* would be predicted to similarly compromise the interaction of SUR-6 with the PP2A core. As *cs24* is nearly wild type for mitotic function but nearly null for Ras signaling function, one possible model is that SUR-6 acts independently of the A and C subunits during mitosis but acts with the A and C subunits during Ras signaling. An alternative model is that SUR-6 acts with the A and C subunits during both processes, but that only very low levels of SUR-6/PP2A are required for mitotic function. The latter model is supported by the fact that maternally rescued *sur-6(sv30)* mutants show few or no mitotic defects during larval development (presumably owing to perdurance of low levels of maternal product), and by the fact that reducing the *let-92*/PP2A dose by half causes synthetic embryonic lethality in both *sur-6* missense backgrounds. Thus, there appear to be different threshold requirements for the various roles of SUR-6-associated PP2A, with Raf activation being most sensitive to reductions in *sur-6* activity.

Conclusions

In conclusion, we have shown that SUR-6/PR55 promotes mitotic progression during embryogenesis and Raf activation during vulval induction and that it cooperates with LET-92/PP2A-C during both processes. Therefore, SUR-6/PP2A probably dephosphorylates mitotic and Ras pathway substrates. Thus far, the identification of PP2A targets has been hampered by the very broad specificity of PP2A in vitro and the widespread requirement for PP2A in vivo. The fact that *sur-6* null mutants show specific phenotypes makes *C. elegans* a useful genetic model system in which to test candidate PP2A targets and identify those that are functionally relevant.

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