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A component of the transcriptional mediator complex inhibits RAS-dependent vulval fate specification in *C. elegans*

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SUMMARY

Negative regulation of receptor tyrosine kinase (RTK)/RAS signaling pathways is important for normal development and the prevention of disease in humans. We have used a genetic screen in *C. elegans* to identify genes that antagonize the activity of activated LET-23, a member of the EGFR family of RTKs. We identified two loss-of-function mutations in *dpy*-22, previously cloned as *sop-1*, that promote the ability of activated LET-23 to induce ectopic vulval fates. DPY-22 is a glutamine-rich protein that is most similar to human TRAP230, a component of a transcriptional mediator complex. DPY-22 has previously been shown to regulate WNT responses through inhibition of the β-catenin-like protein BAR-1. We provide evidence that DPY-22 also inhibits RAS-dependent vulval fate specification independently of BAR-1, and probably regulates the activities of multiple transcription factors during development. Furthermore, we demonstrate that although inhibition of BAR-1-dependent gene expression has been shown to require the C-terminal glutamine-rich region, this region is dispensable for inhibition of RAS-dependent cell differentiation. Thus, the glutamine-rich region contributes to specificity of this class of mediator protein.

Key words: EGF, RAS, LET-23, SOP-1, DPY-22, Mediator, Vulva, *C. elegans*

INTRODUCTION

Activation of signal transduction pathways by growth factors is frequently used in development as a mechanism to specify distinct cell fates. In the case of the *C. elegans* hermaphrodite, vulval cell fate specification (reviewed by Greenwald, 1997; Sternberg and Han, 1998) begins at the end of the second larval stage with the secretion of LIN-3 (Hill and Sternberg, 1992), an EGF-like growth factor, from the gonadal anchor cell. Six Pn.p cells, P3.p-P8.p, comprise the vulval precursor cells (VPCs). These cells express LET-23 (Aroian et al., 1990; Kaech et al., 1998; Simske et al., 1996), an EGF receptor-like protein, and are competent to respond to overexpressed LIN-3 by adopting vulval fates (Hill and Sternberg, 1992; Katz et al., 1995). However, in the presence of physiological levels of LIN-3, only P6.p adopts a primary vulval fate. This response is transduced through LET-60 (RAS) (Beitel et al., 1990; Han and Sternberg, 1990) and SUR-1/MPK-1 (MAP kinase) (Lackner et al., 1994; Wu and Han, 1994), and is accompanied by the activation of LIN-12, a NOTCH-like molecule (Yochem et al., 1988), on adjacent P5.p and P7.p, which causes these cells to adopt secondary vulval fates (Simske and Kim, 1995). The remaining VPCs, P3.p, P4.p and P8.p, do not adopt vulval fates, and fuse with the hypodermal syncytium, hyp7.

Given the likely diffusible nature of LIN-3, mechanisms must exist to ensure that normally only a subset of the VPCs responds to the growth factor. A ligand-independent gain-of-function allele of *let-23*, *sa62*, confers ectopic vulval fate transformations in P3.p and P4.p at high frequency (Katz et al., 1996), indicating that receptor activation is sufficient to drive cell differentiation. Under physiological conditions, positional selectivity for cell fate determination might be achieved by dependency on a threshold level of LET-23 pathway activation, which is normally achieved only by the VPC closest to the anchor cell, the source of LIN-3. Consistent with this model, P6.p is positioned closest to the anchor cell and invariantly responds to LIN-3.

Genetic studies of negative regulation of vulval development indicate that additional mechanisms operate to ensure the selective response of P6.p to LIN-3. Although ectopically activated LET-23 induces vulval fate transformations at high frequency in P3.p and P4.p, the posteriorly expressed *mab-5* homeobox gene inhibits this response in the most posterior Pn.p cell, P8.p (Clandinin et al., 1997). Another mechanism of restricting the response to LIN-3 involves two classes of genes that function redundantly to inhibit vulval fates. Animals that harbor loss-of-function mutations both in a class A and class B ‘synmuv’ gene display a synthetic multivulva phenotype because of the adoption of vulval fates by most of the VPCs (Ferguson and Horvitz, 1989). This vulval induction is independent of the LIN-3-producing anchor cell, but dependent on LET-23 and its downstream effectors (Ferguson et al., 1987; Huang et al., 1994; Lu and Horvitz, 1998), raising the possibility that this pathway exists to repress low, but functional, levels of ligand-independent activity by the LET-23 pathway. Molecular identification of some of the synmuv genes

and RNA interference experiments suggest that this pathway comprises components of a histone deacetylase complex, which represses LET-23-dependent gene expression (Chen and Han, 2001; Lu and Horvitz, 1998; Solari and Ahringer, 2000).

Several genes have been identified whose properties do not fully resemble the synmuv genes, but nevertheless, function as negative regulators of vulval induction. These include *unc-101*(AP47 medium chain of trans-Golgi AP-1 complexes) (Lee et al., 1994), *sli-1*(c-CBL) (Yoon et al., 1995), *gap-1* (Hajnal et al., 1997), *ark-1* (ACK-related tyrosine kinase) (Hopper et al., 2000) and *lip-1* (MAP kinase phosphatase) (Berset et al., 2001). Mutations in these genes suppress loss-of-function mutations in the *let-23* pathway that cause vulvaless phenotypes, and in different genetic backgrounds, they enhance the frequency of multivulva phenotypes, indicating they affect all six VPCs, similar to the synmuv genes. These negative regulators may function to raise the requirement for the amount of LET-23 pathway activity necessary to drive a functional response.

To address whether these are the only mechanisms and points of negative regulation of the response to LIN-3, we performed a genetic screen for mutations that enhance the frequency of ectopic vulval fate transformations in the presence of gain-of-function *let-23(sa62)*. We isolated two new alleles of *sop-1* (Zhang and Emmons, 2000), which has recently been found to be allelic to the older locus, *dpy-22* (Meneely and Wood, 1987) (H. Sawa, personal communication). DPY-22 is most closely related to human TRAP230 (Ito et al., 1999; Nagase et al., 1996; Philibert et al., 1998), a component of the transcriptional mediator complex (Ito et al., 1999), and has been shown to be an inhibitor of WNT-dependent ray formation in the *C. elegans* male tail (Zhang and Emmons, 2000). We describe some of the phenotypes of our new *dpy-22* alleles, and present evidence that DPY-22 also is an inhibitor of RAS-dependent vulval fate specification, independent of its role in regulating WNT signaling.

MATERIALS AND METHODS

Strains and mutagenesis

C. elegans were cultured at 20°C using standard protocols (Brenner, 1974). Alleles used in this work were: *pry-1(mu38)* (Maloof et al., 1999) on LGI; *let-23(sy1)* (Aroian and Sternberg, 1991), *let-23(sa62gf)* (Katz et al., 1996) and *unc-4(e120)* (White et al., 1992) on LGII; *pha-1(e2123ts)* (Granato et al., 1994) on LGIII; *let-60(n1046gf)* (Beitel et al., 1990; Ferguson and Horvitz, 1985), *unc-31(e169)* (Avery et al., 1993) and *dpy-4(e1166)* (Cox et al., 1980) on LGIV; *him-5(e1490)* (Hodgkin et al., 1979) on LGV; and *lon-2(e678)* (Brenner, 1974), *bar-1(ga80)* (Eisenmann et al., 1998), *bar-1(mu63)* (Maloof et al., 1999), *dpy-6(e14)* (Brenner, 1974), *dpy-22(bx93)* (Zhang and Emmons, 2000), *egl-15(n484)* (Trent et al., 1983) and *unc-9(e101)* (Brenner, 1974) on LGX. Genetic balancers used were: *mnC1 [dpy-10(e128) unc-52(e444)]* (Herman, 1978; Sigurdson et al., 1984) on LGII; *nT1[let(m435)]* on LGIV and LGV (Rogalski and Riddle, 1988). All *let-23(sa62)* strains carried the linked mutation *unc-4(e120)*, and heterozygous *let-23(sa62)*/+ strains were balanced with *mnC1*. *let-60(n1046)*/+ strains carried the linked marker *unc-31(e169)* and were balanced by *nT1[let(m435)]*.

bar-1(mu63) was previously reported to have a mutation causing a L130F change (Maloof et al., 1999), which we did not detect in the extant *bar-1(mu63)* strains. In order to determine the molecular lesion in *bar-1(mu63)*, we amplified 1 kb regions of *bar-1* genomic DNA from *bar-1(mu63)* animals by PCR, and directly sequenced the products. Primer sets used included BAR1-6 5′-ttc agt tct act tgt cta ttg gtg tgc-3′ and BAR1-7 5′-cac atg gta gtc cgc gac ttg tac-3′; BAR1-8 5′-cga gaa ttg acc agc tcc aga aga g-3′ and BAR1-9 5′-gc tgc tta ctg atg aag ccg gtg-3′; and BAR1-10 5′-gc ttt gtg cac aac ctc ctg taa g-3′ and BAR1-11 5′-ct ctt cat ccg gca gac aaa tcg-3. After sequencing 36% of the *bar-1* genomic locus, we identified a C to T mutation at position 39108 of C54D1 in *bar-1(mu63)* animals. This change was detected on both strands, and not in N2 animals. This mis-sense mutation is predicted to cause a G524D change in the BAR-1 protein. Linkage of *bar-1(mu63)* to *dpy-22(sy622)* was confirmed by DNA sequencing. Linkage of *bar-1(ga80)* to *dpy-22(sy622)* was confirmed by PCR and digestion with *Mse*I, which detects the *Mse*I restriction site created by the *ga80* C to T point mutation.

let-23(sa62); him-5(e1490) animals were subjected to standard mutagenesis with ethylmethanesulfonate (Brenner, 1974). One thousand F1 offspring were picked to individual plates, and those segregating adult males with ventral protrusions were saved.

Molecular biology

Full-length *dpy-22* used for PCR-based rescue was amplified by PCR (Expand Long, Roche) from positions 21115 to 6068 of cosmid F47A4 with the primers 5′-gtc ccg tta tga taa cgt atc tcc aag-3′ and 5′-caa gcg tta tct tga tga cgc ggt c-3′. The PCR fragment was injected at 10 ng/μl with pPD118.33 (*myo-2::gfp*) (10 ng/μl) and pBSSK (Stratagene) (160 ng/μl) into *dpy-6(e14) dpy-22(sy622); stDp2* animals. Rescuing arrays were subsequently crossed into *dpy-22(sy622)* single mutants.

A full-length *dpy-22* gene spanning 20759 to 6464 of cosmid F47A4 was reconstructed in pBR322 as follows. First, an *Xho*I fragment from F47A4 (20759-10290) was cloned into the *Sal*I site of pBR322 to yield pBRF47A4Xh. This plasmid was digested with *Age*I, and an *Age*I fragment from F47A4 (10406-6464) was introduced into this vector, to generate pBRsop1FL, which harbors a full-length *dpy-22* gene. *dpy-22::gfp*, *dpy-22 2548::gfp* and *dpy-22 2141::gfp* transgenes were constructed using overlap extension PCR (High Fidelity, Roche) to make C-terminal in-frame translational fusions between appropriately truncated *dpy-22* fragments and *gfp*. In all cases, codons encoding two glycine residues were placed between the two genes, and the fusions were made to the codon encoding the first Ser residue of GFP. *dpy-22* fragments were amplified by PCR from F47A4 using the 5′ primer sop1-17 5′-ct tat gtt cca cgg tat cat caa tcc-3′ and the 3′ primer sop1-28 5′-c ttc tcc ttt act tcc tcc gta ctg att tgg tgg ttg ttg gtt g-3′; sop1-26 5′-c ttc tcc ttt act tcc tcc ttt ctg ctg ctc cac aag ttg ttg atg g-3′; or sop1-35 5′- c ttc tcc ttt act tcc tcc gaa cat tct gaa ctt cca tcc gcc-3′, for *dpy-22::gfp*, *dpy-22 2548::gfp* and *dpy-22 2141::gfp*, respectively. *gfp* fragments included the *unc-54* 3′ untranslated region, and were amplified by PCR from pPD95.79 with the 5′ primers sop1-34 5′-aat cag tac gga gga agt aaa gga gaa gaa ctt ttc act gg-3′, sop1-33 5′-cag cag aaa gga gga agt aaa gga gaa gaa ctt ttc act gg-3′ or sop1-32 5′-aga atg ttc gga gga agt aaa gga gaa gaa ctt ttc act gg-3′, for *dpy-22::gfp*, *dpy-22 2548::gfp*, and *dpy-22 2141::gfp*, respectively, and the 3′ primer unc54-5 5′-a taa gaa tgc ggc cgc aaa cag tta tgt ttg gta tat tgg gaa tg-3′. The individual fragments were purified by agarose gel electrophoresis, mixed in the appropriate combinations, and then subjected to PCR in the presence of the 5′ primer sop1-17, and the 3′ primer unc54-5. The *dpy-22::gfp* fusion was digested with *Not*I and cloned into *Not*I-digested pBRsop1FL. *dpy-22 2548::gfp* and *dpy-22 2141::gfp* truncations were digested with *Bst*EII and *Not*I, and cloned into *Bst*EII/*Not*I-digested pBRsop1FL. *dpy-22::gfp* and *dpy-22 2548::gfp* transgenes were injected at 10 ng/μl with pPD118.33 (*myo-2::gfp*) (10 ng/μl) and 160 ng/μl of pBSSK into *dpy-6(e14) dpy-22(bx93)/dpy-22(sy622)* animals. *dpy-22 2141::GFP* was injected at 12.5 ng/μl with pBX-1 (Granato et al., 1994) (100 ng/μl) and pBSSK (37.5 ng/μl) into *pha-1(e2123ts)* animals.

Identification of *sy622* and *sy665* as alleles of *dpy-22*/*sop-1*

sy622 and *sy665* were placed on LGX by crossing N2 males into mutant hermaphrodites and observing that 100% of F1 males were small/dumpy (Dpy) and had abnormal tails (Mab). Three-factor mapping was carried out following the small/Dpy and egg-laying-defective (Egl) phenotypes of *sy622*. *sy622* was placed between *lon-2* and *unc-9* as 6/11 Lon non-Unc and 4/11 Unc non-Lon recombinants picked up the mutation. *sy622* was placed between *dpy-6* and *unc-9* as 6/13 Dpy non-Unc and 8/10 Unc non-Dpy recombinants picked up *sy622*. *sy622* was placed between *lon-2* and *egl-15* as 23/26 Lon-non-Egl and 4/33 Egl non-Lon recombinants picked up *sy622*. Using *sy622 egl-15* double mutants and CB4856, a Hawaiian isolate of *C. elegans*, Egl-non *sy622* recombinants were generated that allowed us to analyze the positions of crossovers by the absence or presence of single nucleotide polymorphisms (SNPs) from CB4856 (Wicks et al., 2001). *sy622* was mapped to the left of the SNP at 36555 in F47A4. Using *dpy-6 sy622* double mutants and CB4856, and picking Dpy non-*sy622* and *sy622* non-Dpy recombinants, *sy622* was mapped to the right of the SNP at 19169 of F15G9. Owing to the unhealthiness of *sy622* animals, cosmids from this region initially were coinjected with pPD118.33 (*myo-2::gfp*) and pBX-1 into *pha-1(e2123ts)* animals, and stable extrachromosomal arrays were crossed into *sy622* animals to test for rescue. Initial attempts at rescue failed using this strategy. Later, cosmid F47A4 (30 ng/μl) was co-injected with pPD118.33 (*myo-2::gfp*)(10 ng/μl) and pBSSK (140 ng/μl) into *pal-1(e2091); him-5(e1490); dpy-22(bx93)* animals. Arrays conferring functional rescue in this background were crossed into *sy622* animals and found to rescue all of the *sy622* phenotypes. *dpy-22* genomic DNA was amplified in 1 kb pieces from *sy622* and *sy665* worms, and the products directly sequenced. Mutations were confirmed by sequencing both strands, and comparing the sequencing data from N2, *sy622* and *sy665* animals in the appropriate regions.

RNAi

Exon 17 from *dpy-22* was amplified by PCR from the cosmid F47A4 with the primers 5′-tta ata cga ctc act ata ggg aga cat tcg aac tag ctc cag aga aac-3′ and 5′-tta ata cga ctc act ata ggg aga atc aaa tgg gta ctt ccc agc ttc-3′, which introduce a T7 bacteriophage promoter at both ends of the fragment. An intronless GFP gene was amplified from the plasmid pPD79.44, with the primers 5′-tta ata cga ctc act ata ggg aga tga gta aag gag aag aac ttt tca c-3′ and 5′-tta ata cga ctc act ata ggg aga cta ttt gta tag ttc atc cat gcc atg-3′, which also add a T7 promoter to both ends of the PCR product. dsRNA was synthesized in vitro using the MEGAscript T7 kit (Ambion). The presence of dsRNA was confirmed by agarose gel electrophoresis, and quantified by spectrophotometry. L1 stage hermaphrodites were incubated in 12 μl M9 buffer containing 1.5 mg/ml total RNA and OP50 (A600 nm=1.0) for 24 hours at 20°C. After incubation, worms were recovered and placed on standard NG plates with OP50, and allowed to develop to the mid-L4 stage, at which time they were examined by Nomarski.

Vulval induction assay and gonad ablations

Vulval development was scored during the L4 stage under Nomarski optics (Sternberg and Horvitz, 1986). Nuclei in the ventral region of the worm that were not of hypodermal, neuronal or muscle descent were counted. In wild-type animals, 22 nuclei arise from vulval fates. The number of vulval nuclei is used to extrapolate how many of the Pn.p cells were induced to adopt vulval fates. A vulval precursor cell (VPC) in which both daughter cells divide one more time, and both granddaughters divide to generate seven or eight great granddaughters and no hypodermal tissue, is scored as 1.0 cell induction. A VPC in which one daughter fuses with the hypodermis, and one daughter continues to divide over the next two generations, resulting in four great granddaughter cells is scored as 0.5 cell induction. In wild-type animals, P5.p, P6.p and P7.p each undergo the equivalent of 1.0 cell induction, whereas the other Pn.p cells do not adopt vulval fates, resulting in an overall cell induction of 3.0. Animals displaying a cell induction of more than 3.0 are multivulva, and animals with a cell induction less than 3.0 are vulvaless. Laser ablations were conducted using a standard protocol (Bargmann and Avery, 1995). Gonadal cells (Z1, Z2, Z3 and Z4) were ablated during the L1 stage.

RESULTS

sy622 and *sy665* promote vulval fate specification

To identify new negative regulators of LET-23-dependent vulval fate specification, we employed a screen using a gain-of-function allele of *let-23*, *sa62*. *let-23(sa62)* encodes a receptor with a mis-sense mutation changing C359Y in the extracellular domain (Katz et al., 1996). In wild-type hermaphrodites, only three VPCs, P5.p-P7.p, adopt vulval fates, whereas in 98% of hermaphrodites homozygous for *let-23(sa62)*, one additional Pn.p, usually P3.p and/or P4.p, adopts a vulval fate (Table 1; see Table 4) (Katz et al., 1996). This ectopic induction, as well as the normal induction displayed by P5.p-P7.p is independent of the LIN-3-producing anchor cell in the gonad, suggesting that when expressed at these levels, the receptor is ligand independent (see Table 4) (Katz et al., 1996). Male gonads do not give rise to an anchor cell, and their Pn.p cells do not normally adopt vulval fates. However, despite the ligand-independence of LET-23(C359Y), this activated receptor is unable to cause male Pn.p cells to adopt vulval fates at high frequency (data not shown). We hypothesized that vulval tissue formation in homozygous *let-23(sa62)* males could be used as a sensitized assay to identify inhibitors of vulval fate specification. We mutagenized *let-23(sa62); him-5(e1490)* hermaphrodites with EMS, and looked for F1 animals that segregated a high percentage of progeny males exhibiting vulval tissue. From a pilot screen of 1000 haploid genomes, three mutations were isolated. Two alleles, *sy622* and *sy665*, resembled each other in that mutant worms shared additional phenotypes (see below), whereas the third mutation seemed to affect only Pn.p fates.

To determine whether *sy622* and *sy665* affected vulval fate specification only in male Pn.p cells, we introduced these mutations into a sensitized hermaphrodite background. Hermaphrodites heterozygous for *let-23(sa62)* have mostly wild-type vulvae, but occasionally some animals display ectopic vulval tissue (Table 1). *sy622* and *sy665* enhance the frequency of ectopic vulval fate specification in *let-23(sa62)*/+ animals (Table 1), indicating that the mutations also affect the response of hermaphrodite Pn.p cells to activated LET-23, and that some regulators of LET-23 signaling are shared between male and hermaphrodite Pn.p cells.

We tested whether the multivulva-enhancing effect of *sy622* was unique to *let-23(sa62)*, or reflected an interaction with activated RAS. *let-60(n1046)* is a gain-of-function *let-60* allele that encodes a RAS protein with a G13E change (Beitel et al., 1990). Seventy-three percent of *let-60(n1046)* homozygotes display ectopic vulval cell fate differentiation (Table 1). However, similar to *let-23(sa62)*/+ animals, *let-60(n1046)*/+ heterozygotes have mostly wild-type vulvae (Table 1). We therefore used *let-60(n1046)*/+ as a sensitized background to examine specifically the interaction of *sy622* with activated RAS. *sy622* enhanced ectopic vulval fate specification in a *let-*

Table 1. *sy622* and *sy665* promote vulval development

Relevant genotype	% Vul*	% P6.p induction†	% Muv‡	VPC induction§	*n*¶	*P* value**
N2	0	100	0	3.0±0.0	31	
let-23(sa62)	0	100	98	4.3±0.6	42	
let-23(sa62)/+	0	100	4	3.1±0.3	26	
sy622	0	100	3	3.0±0.2	35	
sy665	0	100	2	3.0±0.0	50	
let-23(sa62)/+; sy622	0	100	96	4.9±0.8	23	<0.000001 versus *let-23(sa62)/+*
let-23(sa62)/+; sy665	0	100	95	4.1±0.7	20	0.000001 versus *let-23(sa62)/+*
let-23(sa62)/+; dpy-4(e1166)	0	100	15	3.1±0.2	26	0.61 versus *let-23(sa62)/+*
let-60(n1046)	0	100	73	4.0±0.8	22	
let-60(n1046)/+	0	100	4	3.1±0.4	24	
let-60(n1046)/+; sy622	0	100	91	4.2±0.8	23	<0.000001 versus *let-60(n1046)/+*
lin-3(n378)	96	35	0	0.7±1.0	51	
lin-3(n378); sy622	84	86	0	1.6±0.9	37	0.00002 versus *lin-3(n378)*
lin-3(n378); sy665	78	76	0	1.6±1.0	45	0.00005 versus *lin-3(n378)*
let-23(sy1)	100	29	0	0.5±0.9	24	
let-23(sy1); sy622	75	90	0	2.1±0.9	20	<0.000001 versus *let-23(sy1)*
let-23(sy1); sy665	62	90	5	2.3±0.8	21	<0.000001 versus *let-23(sy1)*

*Vulvaless. Percentage of animals that have less than three VPCs adopting vulval fates.
†Percentage of animals with P6.p adopting a vulval fate.
‡Multivulva. Percentage of animals that have more than three VPCs adopting vulval fates.
§Average number of VPCs that adopt vulval fates.
¶Number of animals assayed.
***P* values were calculated for VPC induction using Student's *t*-test.

Table 2. *sy622* and *sy665* animals have multiple phenotypes

Relevant genotype	Body length*	% Egl†	% Mab‡	*P* value§
N2	87±5 (*n*=23)	0 (*n*=30)	3 (*n*=34)	
sy622	61±6 (*n*=23)	92 (*n*=61)	95 (*n*=39)	<0.00001
sy665	64±4 (*n*=22)	100 (*n*=36)	100 (*n*=35)	<0.00001
let-23(sa62)/+; dpy-4(e1166)	58±3 (*n*=20)	nd	nd	<0.00001
sy622; syEx538 [F47A4]	88±5 (*n*=20)	8 (*n*=24)	0 (*n*=25)	0.47
sy622; syEx539 [dpy-22 PCR]	84±5 (*n*=20)	11 (*n*=28)	9 (*n*=23)	0.09
dpy-22(bx93)	83+4 (*n*=20)	0 (*n*=30)	3 (*n*=33)	0.002
dpy-22(sy622)/dpy-22(bx93)	81±5 (*n*=18)	13 (*n*=23)	NA	0.0007
bar-1(mu63) dpy-22(sy622)	65±5 (*n*=20)	93 (*n*=30)	100 (*n*=25)	0.018
dpy-22(sy622); syEx554 [dpy-22:: gfp]	93±4 (*n*=23)	0 (*n*=39)	4 (*n*=27)	0.00005
dpy-22(sy622); syEx558 [dpy-22 2548:: gfp]	90±5 (*n*=26)	0 (*n*=33)	4 (*n*=25)	0.07

*Body length of young adults (×0.01 mm).
†Percentage of animals that are egg-laying defective.
‡Percentage of males that have abnormal ray development.
§*P* values were calculated for body length compared with N2 using Student's *t*-test.
n, number of animals assayed; NA, not applicable; nd, not determined.

60(n1046)/+ background (Table 1), consistent with the *sy622* mutation increasing the response to RAS activation.

We also tested if *sy622* and *sy665* could affect vulval fate specification in the central VPCs, P5.p-P7.p, which are normally specified to form vulval tissue. We found that both *sy622* and *sy665* could restore the ability of P6.p to adopt a vulval fate in the presence of the *lin-3(n378)* or *let-23(sy1)* reduction-of-function mutations, which reduce RAS signaling (Table 1). However, many of these double mutants still do not display wild-type vulval induction, indicating *sy622* and *sy665* do not bypass the requirements for RAS signaling.

sy622 and *sy665* have additional phenotypes

In addition to enhancing vulval fate specification in the presence of activated LET-23, the *sy622* and *sy665* mutations cause other phenotypes. *sy622* and *sy665* hermaphrodites have a 90% penetrant egg-laying defect (Table 2), and, as young adults, are dumpyish and only about 70% of the size of wild-type animals (Fig. 1A-C; Table 2). In addition, 95% of *sy622* and *sy665* adult males have abnormal ray development in the tail (Fig. 1D-F; Table 2). Given the shortened body length of *sy622* and *sy665* animals, we asked whether the effect on ectopic vulval fate specification might indirectly result from a reduced distance between the anchor cell and P3.p, P4.p and P8.p. We, therefore, introduced the *dpy-4(e1166)* mutation, which comparably reduces body length (Table 2), into *let-23(sa62)/+* animals. Unlike *sy622* and *sy665*, *dpy-4(e1166)* did not significantly enhance ectopic vulval fate specification in this background, indicating that the effect of *sy622* and *sy665* on vulval induction is not due to a general reduction in body size (Table 1).

sy622 and *sy665* are strong loss-of-function alleles of *dpy-22/sop-1*

Based on the similarity in phenotypes between *sy622* and *sy665*, and their common linkage to the X chromosome, we tested whether they were allelic. These recessive alleles failed to complement each other for egg-laying and body size defects, suggesting that they define a single locus (data not shown). We three-factor mapped *sy622* between *dpy-6* and *egl-15* on the genetic map, and using single nucleotide polymorphisms in CB4856, a Hawaiian isolate of *C. elegans*, we placed *sy622* between the cosmids F15G9 and F47A4 on the physical map. Cosmid F47A4 rescued the egg-laying, body size and male tail defects (Table 2). A PCR fragment encompassing the complete predicted coding region of only *F47A4.2*, *sop-1* (Zhang and Emmons, 2000) (Fig. 2A), also rescued the egg-laying, body size and male tail defects (Table 2). *F47A4.2/sop-1* has recently been found to be allelic to an older genetic locus, *dpy-22* (Meneely and Wood, 1987) (H. Sawa, personal communication). When crossed into a *let-23(sa62)/+; sy622* background, extra-chromosomal arrays containing either F47A4 or the *dpy-22* PCR product suppressed the ectopic vulval fate specification observed in these animals (Table 3). These results suggest that all of the *sy622* phenotypes result from mutation of a single gene, *dpy-22*. We then sequenced *dpy-22* genomic DNA from *sy622* and *sy665* animals. We found that relative to wild-type animals, *sy622* animals harbored a C to T mutation at position 13,100 of F47A4, which changes a CAG glutamine codon in exon 12 to an Amber STOP codon. *sy665* animals harbored a C to T mutation at position 11,718 of F47A4, which changes a CAA glutamine codon in exon 13 to a TAA Ochre STOP codon. The mutant DNA in *sy622* and *sy665* animals is predicted to truncate the DPY-22 protein after amino acids 1697 and 2141, respectively (Fig. 2B).

dpy-22 was cloned as *sop-1*, by virtue of the isolation of non-Dpy alleles that suppress the ray loss phenotype conferred by a regulatory region mutation in the homeobox gene *pal-1* (Zhang and Emmons, 2000). The *dpy-22* alleles that suppress *pal-1(e2091)* consist of one splicing mutation and three nonsense mutations, with the strength of the allele correlated with the extent of the predicted C-terminal truncation. *dpy-22(bx93)* and *dpy-22(bx92)* are the strongest alleles, and they are expected to make proteins that are truncated after amino acids 2548 and 3165, respectively. RNAi experiments indicate that all of these *dpy-22* alleles are reduction-of-function mutations, raising the possibility that *dpy-22(sy622)* and *dpy-22(sy665)* are more severe loss-of-function alleles, because they would cause even earlier truncations than would *dpy-22(bx93)*. Consistent with this notion, recent RNAi experiments against *dpy-22*, and double mutant analyses using the *dpy-22* alleles that suppress *pal-1(e2091)* and either *unc-37* or *sur-2* also have revealed a ray loss phenotype (Zhang and Emmons, 2002), similar to that observed in *dpy-22(sy622)* and *dpy-22(sy665)* single mutants (Fig. 1). This observation suggests that ray development is ultimately compromised when DPY-22 pathway activity is reduced below a certain threshold. In addition, we find that the loss-of-function allele *dpy-22(bx93)* weakly promotes ectopic vulval fate transformations in a *let-23(sa62)/+* background (Table 3). Moreover, *dpy-22(bx93)* fails to complement *dpy-22(sy622)* for enhancing ectopic vulval fate specification in the presence of *let-23(sa62)/+* (Table 3), providing further evidence that DPY-22 negatively regulates vulval development.

To examine directly the effects of reducing DPY-22 levels on vulval development, we used RNAi against *dpy-22*. In an

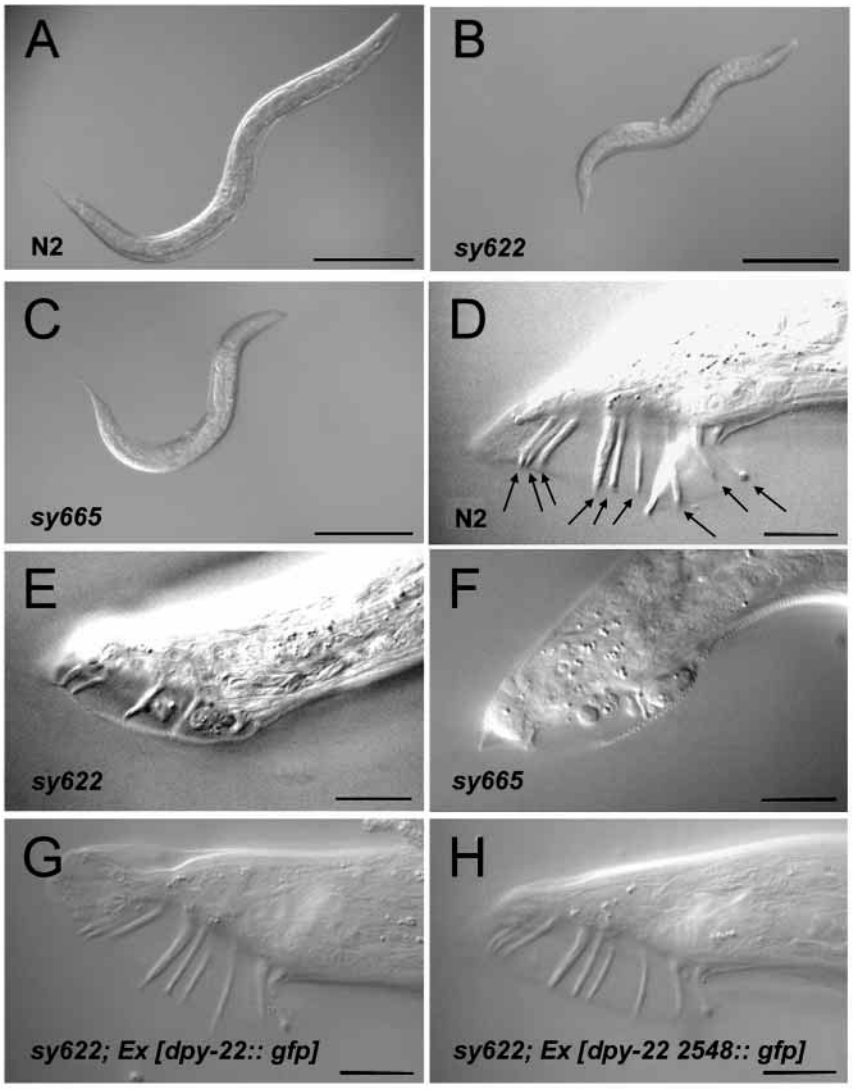

Fig. 1. *sy622* and *sy665* animals have multiple phenotypes. Scale bars: 100 μm in A-C; 20 μm in D-H. All animals are 1-day-old young adults. (A) Wild-type hermaphrodite. (B) *sy622* hermaphrodite. (C) *sy665* hermaphrodite. (D) Wild-type male tail. Arrows indicate wild-type rays. (E) *sy622* male tail. (F) *sy665* male tail. (G) A *sy622* male harboring a rescuing *dpy-22::gfp* transgene that produces a full-length DPY-22::GFP translational fusion protein. (H) A *sy622* male harboring a rescuing *dpy-22 2548::gfp* transgene, which produces a translational fusion between the first 2548 amino acids of DPY-22 to GFP.

Table 3. DPY-22 is an inhibitor of vulval development

Relevant genotype	% Vul*	% Muv†	VPC induction‡	*n*§	*P* value¶
N2	0	0	3.0±0.0	31	
let-23(sa62)	0	98	4.3±0.6	42	
let-23(sa62)/+	0	4	3.1±0.3	26	
sy622	0	3	3.0±0.2	35	
let-23(sa62)/+; sy622	0	96	4.9±0.8	23	<0.000001 versus *let-23(sa62)/+*
let-23(sa62)/+; sy622; syEx538 [F47A4]	0	0	3.0±0.0	24	0.33 versus *let-23(sa62)/+*
let-23(sa62)/+; sy622; syEx539 [dpy-22 PCR]	0	14	3.1±0.2	22	0.88 versus *let-23(sa62)/+*
dpy-22(bx93)	0	0	3.0±0.0	23	
let-23(sa62)/+; dpy-22(bx93)	0	40	3.3±0.3	20	0.032 versus *let-23(sa62)/+*
let-23(sa62)/+; dpy-22(sy622)/+	0	15	3.1±0.3	26	0.42 versus *let-23(sa62)/+*
let-23(sa62)/+; dpy-22(sy622)/dpy-22(bx93)	0	71	3.7±0.6	24	0.00004 versus *let-23(sa62)/+*
let-23(sy1)	100	0	0.5±0.9	24	
let-23(sy1); gfp RNAi	100	0	0.2±0.4	41	
let-23(sy1); dpy-22 RNAi	79	6	1.9±0.9	34	<0.000001 versus *gfp RNAi*
let-23(sa62)/+; dpy-22(sy622); syEx554 [dpy-22:: gfp]	0	13	3.1±0.3	23	0.55 versus *let-23(sa62)/+*
let-23(sa62)/+; dpy-22(sy622); syEx558 [dpy-22 2548:: gfp]	0	15	3.1±0.3	20	0.60 versus *let-23(sa62)/+*

*Vulvaless. Percentage of animals that have less than three VPCs adopting vulval fates.
†Multivulva. Percentage of animals that have more than three VPCs adopting vulval fates.
‡Average number of VPCs that adopt vulval fates.
§Number of animals assayed.
¶*P* values were calculated for VPC induction using Student's *t*-test.

otherwise wild-type background, *dpy-22* RNAi did not affect vulval development (data not shown). However, in a sensitized background consisting of the *let-23(sy1)* loss-of-function mutation, *dpy-22* RNAi could partially suppress the vulvaless phenotype of *let-23(sy1)*, similar to the *dpy-22* alleles *sy622* and *sy665* (Table 3). By contrast, control *gfp* dsRNA did not suppress *let-23(sy1)* (Table 3). Together, these data indicate that *sy622* and *sy665* are stronger loss-of-function alleles of *dpy-22*.

dpy-22 is expressed in vulval precursor cells and is mislocalized in *dpy-22(sy665)* animals

DPY-22 is most closely related to human TRAP230 (Ito et al., 1999; Nagase et al., 1996; Philibert et al., 1998), a component of human mediator complexes, and KOHTALO (Treisman, 2001), a regulator of cell fate in the *Drosophila* eye. The fly and human proteins have similar length, and display 35% overall identity. DPY-22 is larger than both proteins by ~1000 amino acids, and BLAST analysis indicates that its homology with TRAP230 and KOHTALO is spread over three regions (Fig. 2B). These include small regions of identity at the N and C termini, and a larger region of identity in the middle of the protein. In all three proteins, the C terminus is rich in glutamine. Thirty-three percent of the terminal 781 and 589 amino acids are glutamine in DPY-22 and KOHTALO, respectively, and 41% of the 278 terminal amino acids in TRAP230 are glutamine. Of the previously isolated nonsense mutations in *dpy-22*, *bx93* and *bx92* are the strongest. *bx93* results in the greatest truncation, removing all the amino acids C-terminal to 2548, including the entire glutamine-rich region. As *dpy-22(sy622)* and *dpy-22(sy665)* cause a number of highly penetrant phenotypes (Fig. 1; Tables 1, 2) not observed in the other nonsense mutations, domains N-terminal to the glutamine-rich region must be important for DPY-22 function. The nonsense mutation in *dpy-22(sy622)* would be predicted to truncate DPY-22 in the largest central region of identity; however, *dpy-22(sy665)* is predicted to truncate DPY-22 after amino acid 2141, close to the end of this region (Fig. 2B). This observation suggests that another important functional domain exists between amino acids 2141 and 2548 that does not have identity with TRAP230 or KOHTALO.

PROSITE analysis of DPY-22 indicated the presence of two putative nuclear localization signals in this region. To test whether the stronger phenotypes observed in *dpy-22(sy622)* and *dpy-22(sy665)* animals are correlated with a mislocalized, non-nuclear form of DPY-22, we constructed transgenes in which *dpy-22* DNA was fused in-frame to the green fluorescent protein (GFP) open reading frame (Fig. 2C). *dpy-22* has been reported to be expressed in vulval cells, during the later stages of vulval development (Zhang and Emmons, 2000). We found that a rescuing (see last section of the Results) fusion of wild-type DPY-22 to GFP was expressed in the VPCs, the anchor cell, and hyp7 nuclei at the time of vulval fate specification, and continued to be expressed in these cells throughout vulval development (Fig. 3A-F). Wild-type DPY-22 and DPY-22 truncated after amino acid 2548, as generated in *dpy-22*(*bx93)* mutants, directed nuclear expression of GFP (Fig. 3A-H). By contrast, DPY-22 truncated after amino acid 2141 could not direct GFP into the nucleus, and instead, caused GFP to accumulate in the cytoplasm (Fig. 3I,J). These data indicate that the predicted nuclear localization sequences (NLSs) in this region are functional, and suggest that the severity of the *dpy-22(sy622)* and *dpy-22(sy665)* phenotypes may result from mislocalization of the DPY-22 protein, and/or loss of other functional regions of the protein.

DPY-22 is a gonad-independent inhibitor of vulval fate specification of multiple Pn.p cells

let-23(sa62)/+ and *let-60(n1046)* animals are responsive to

LIN-3 (Chang et al., 2000; Katz et al., 1996; Sundaram and Han, 1995) (Table 3), and suppression of the *lin-3(n378)* and *let-23(sy1)* hypomorphic mutations could in principle occur through elevated production of LIN-3. As DPY-22::GFP is expressed in the anchor cell and the VPCs (Fig. 3), we tested whether *dpy-22* affects vulval fate specification by modulating the production of LIN-3 from the gonad. We ablated the gonadal primordium in *let-23(sa62)/+* and *let-23(sa62)/+; dpy-22(sy622)* early L1 larvae. Gonad-ablated *let-23(sa62)/+* animals displayed very little vulval fate specification in any of the Pn.p cells (Table 4). By contrast, *dpy-22(sy622)* increased the frequency of vulval fate specification in all six VPCs in this background (Table 4), indicating that DPY-22 does not act primarily by regulating LIN-3 production from the gonad, and that the fates of all the VPCs are regulated by DPY-22.

DPY-22-mediated inhibition of vulval-fate specification involves transcription factors other than BAR-1 (β-catenin)

In males, a *bar-1*(β-catenin) null allele, *ga80*, does not affect ray development on its own (Zhang and Emmons, 2000). However, it reduces the degree to which *dpy-22(bx92)* can restore wild-type ray development in a *pal-1(e2091)* mutant background (Zhang and Emmons, 2000). This property has led to the proposal that in the presence of the *pal-1(e2091)* regulatory region mutation, DPY-22 inhibits BAR-1-dependent regulation of the *pal-1* gene (Zhang and Emmons, 2000). WNT/β-catenin signaling also promotes vulval fate specification, parallel to the LET-23-LET-60 pathway. A null mutation in *bar-1* results in an incompletely penetrant vulvaless phenotype (Table 5) (Eisenmann et al., 1998), and a loss-of-function mutation in the axin-like inhibitor of WNT signaling, *pry-1* (Korswagen et al., 2002; Maloof et al., 1999), causes ectopic vulval fate specification (Table 5) (Gleason et al., 2002). Furthermore, hyperactivated WNT signaling can bypass loss-of-function mutations in the *let-23* pathway (Gleason et al., 2002). We therefore tested whether relief of inhibition of BAR-1 might account for the interactions between our *dpy-22* alleles and mutations in the LET-23 pathway during vulval development.

We linked two *bar-1* mutations to *dpy-22(sy622)*. *bar-1(mu63)* encodes a weak loss-of-function mutation (Maloof et al., 1999) that does not confer defects in vulval development on its own (Table 5). The mis-sense mutation in this allele results in a protein with a G524D change (see Materials and Methods). We hypothesized that under conditions where a phenotype was dependent on elevated BAR-1 activity, *bar-1(mu63)* should quantitatively reduce it. Consistent with this notion, *bar-1(mu63)* suppresses the ectopic *mab-5* expression and poly ray phenotypes observed in *pry-1(mu38)* animals (Maloof et al., 1999). As a control, we tested whether *bar-1(mu63)* also could reduce vulval induction in a background where WNT signaling was specifically hyperactivated. We built a *pry-1(mu38); let-23(sy1)* double mutant and, as recently reported (Gleason et al., 2002), found that excess WNT signaling could strongly suppress this *let-23* reduction-of-function allele (Table 5). *bar-1(mu63)* reduced the ability of *pry-1(mu38)* to suppress *let-23(sy1)* (Table 5), indicating that *bar-1(mu63)* can be used to define conditions under which WNT signaling is hyperactivated during vulval development. Although *dpy-22(sy622)* is a weaker suppressor of *let-23(sy1)* than is *pry-1(mu38)*, its ability to suppress the *let-23* mutation was not reduced in the presence of the *bar-1(mu63)* mutation (Table 5). These data suggest that DPY-22 does not interfere with vulval development through inhibition of BAR-1. We also examined the body size, egg-laying behavior and male rays in *bar-1(mu63) dpy-22(sy622)*

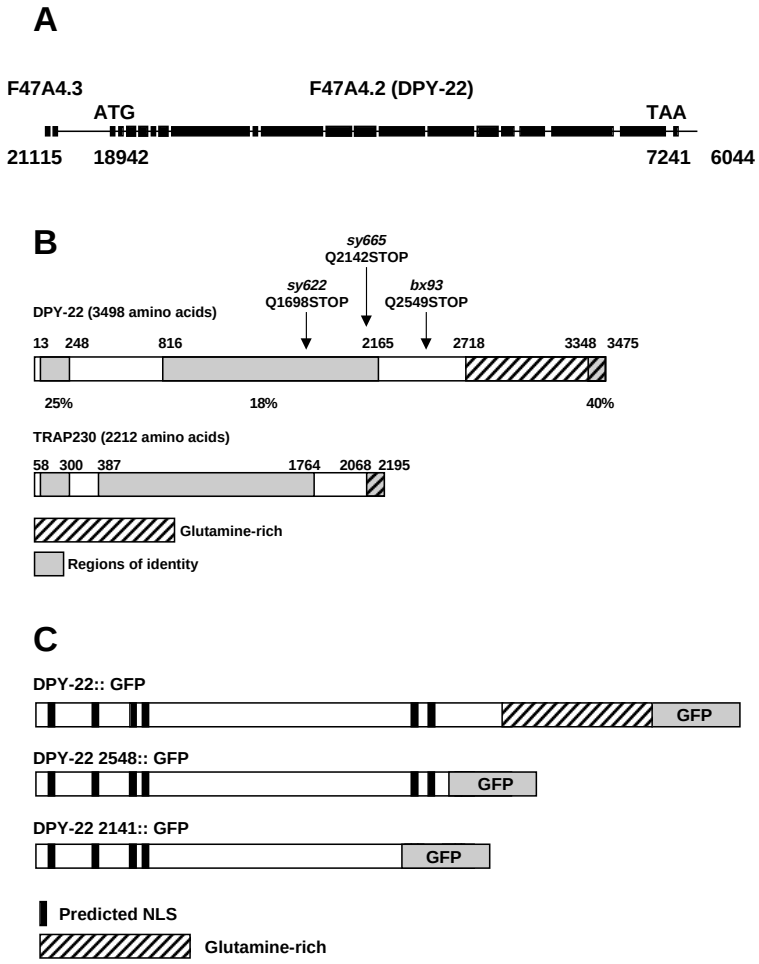

Fig. 2. Structure of injected transgenes, and DPY-22 and TRAP230 proteins. (A) Graphical depiction of the PCR fragment amplified from cosmid F47A4 used for rescue experiments. PCR was used to amplify the region between 21115 and 6044 of F47A4. This region contains the entire predicted *dpy-22* open reading frame (18942-7241) and parts of the last two exons from *F47A4.3*. (B) Structural organization of DPY-22 and TRAP230 proteins. Numbers above the proteins refer to amino acid positions. Hatched area represents a glutamine-rich region. Gray areas represent regions of amino acid identity between DPY-22 and TRAP230, as defined by BLAST, with the percentage identity listed below. The positions of the *dpy-22* nonsense mutations used in this study are indicated. (C) Structure of DPY-22::GFP fusion proteins encoded by transgenic constructs. DPY-22::GFP is a fusion of the full-length wild-type DPY-22 to non nuclear-localized GFP. DPY-22 2548::GFP and DPY-22 2141::GFP are fusions between the first 2548 and 2141 amino acids of DPY-22, respectively, to non nuclear-localized GFP. Black boxes indicate putative nuclear localization sequences predicted by PROSITE. Hatched area represents a glutamine-rich region. Gray areas indicate GFP amino acids.

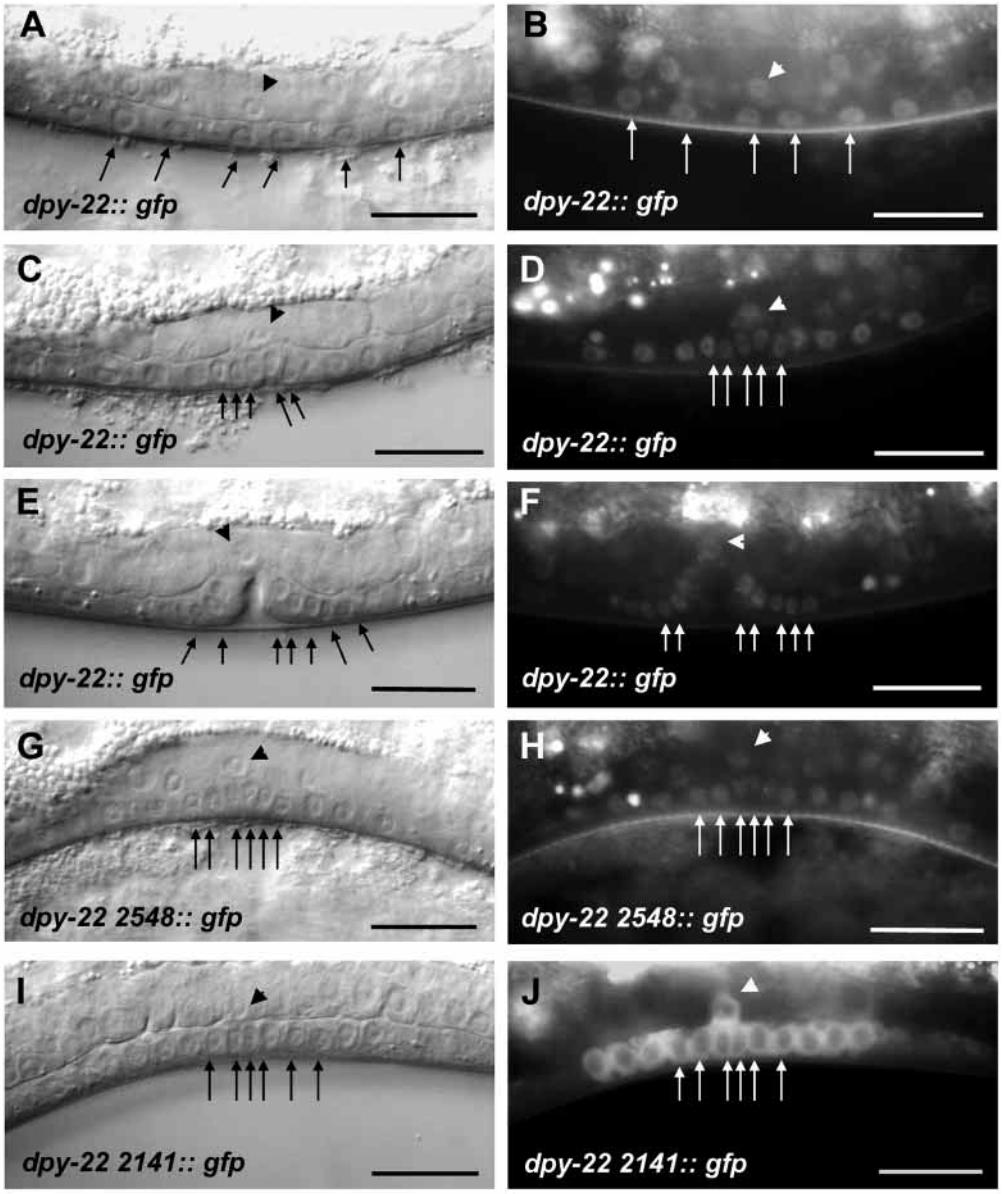

Fig. 3. DPY-22::GFP is expressed in the nuclei of vulval cells, and is mislocalized by the *dpy-22(sy665)* mutation. Left-hand images are Nomarski, and right-hand images are fluorescence. Scale bars: 20 μm. Arrows indicate some of the vulval cells and arrowheads indicate the anchor cells. (A-F) *dpy-22(sy622)* hermaphrodites rescued with a *dpy-22::gfp* transgene that encodes a fusion of full-length wild-type DPY-22 to GFP. (A,B) Two-cell stage at the onset of vulval fate specification. (C,D) Four-cell stage of vulval development. (E,F) Eight-cell stage of vulval development. (G,H) *dpy-22(sy622)* hermaphrodites rescued with a *dpy-22 2548::gfp* transgene, which encodes a fusion of the first 2548 amino acids of DPY-22 to GFP. (I,J) *pha-1(e2123ts)*-rescued animals carrying a *dpy-22 2141::gfp* transgene, which encodes a fusion of the first 2141 amino acids of DPY-22 to GFP. Wild-type DPY-22 and DPY-22 truncated after amino acid 2548 direct GFP to the nucleus, but not DPY-22 truncated after amino acid 2141.

animals, and found none of *dpy-22(sy622)* phenotypes to be suppressed by the *bar-1* mutation (Table 2).

As our data suggested that DPY-22 might primarily inhibit the output of the RAS pathway, rather than the WNT pathway, during vulval development, we tested whether the *dpy-22 (sy622)* mutation was strong enough to compensate for the absence of BAR-1 in *bar-1(ga80)* null mutants. *bar-1(ga80) dpy-22(sy622)* double mutants were sick, but still displayed the *bar-1(ga80)* vulvaless phenotype (Table 5). Because the gain-of-function *let-60(n1046)* allele, which is far stronger than *dpy-22(sy622)* [(Table 1) 73% Muv and 4.0 cell induction versus 3% Muv and 3.0 cell induction, respectively] only suppresses *bar-1(ga80)* to wild-type levels, we hypothesized that failure of *dpy-22(sy622)* to suppress *bar-1(ga80)* might result from insufficient activation of the RAS pathway. Therefore, we used a more sensitive assay to test further whether DPY-22 acts independently of BAR-1 during vulval development. *let-60(n1046gf); bar-1(ga80)* double mutants are mostly wild type and are mutually suppressed with regards to the *let-60(n1046)* multivulva phenotype and the *bar-1(ga80)* vulvaless phenotype (Table 5). As *dpy-22(sy622)* is a strong enhancer of the gain-of-function *let-60(n1046)* allele (Table 1), we asked whether *dpy-22(sy622)* could still enhance activated RAS even in the absence of BAR-1 protein. *let-60(n1046); bar-1(ga80) dpy-22(sy622)* triple mutants were very sick, but survived to L4 and displayed much more vulval induction than did *let-60(n1046); bar-1(ga80)* double mutants (Table 5). The enhanced vulval induction in the triple mutants was abrogated by the presence of the *dpy-22*-rescuing cosmid, F47A4, demonstrating that enhancement in the triple mutant was indeed due to loss of DPY-22 activity (Table 5). These data indicate that DPY-22 can regulate vulval induction independently of any effects on BAR-1 activity.

The C-terminal glutamine-rich region is dispensable for most of the activity of DPY-22

The absence of body size, egg-laying, and ray defects, and the

Table 4. DPY-22 is a gonad-independent inhibitor of vulval fates of multiple VPCs

Relevant genotype	Gonad*	% Vul†	% Muv‡	Frequency of VPC adopting vulval cell fate§						*n*¶
				P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	
N2	+	0	0	0	0	100	100	100	0	31
N2	–	100	0	0	0	0	0	0	0	14
let-23(sa62)	+	0	95	43	95	100	100	100	0	42
let-23(sa62)	–	0	94	71	94	100	94	82	41	17
let-23(sa62)/+	+	0	2	15	15	100	100	100	0	66
let-23(sa62)/+	–	100	0	0	0	4	17	0	0	24
let-23(sa62)/+; dpy-22(sy622)	+	0	96	78	91	96	100	100	57	23
let-23(sa62)/+; dpy-22(sy622)	–	0	50	60	87	100	60	33	33	15

*Absence or presence of gonad. The gonadal primoridum was removed by laser ablation during the L1 larval stage.
†Vulvaless. Percentage of animals that have less than three VPCs adopting vulval fates.
‡Multivulva. Percentage of animals that have more than three VPCs adopting vulval fates.
§Percentage of times that a particular VPC gives rise to some vulval cells.
¶*n*, number of animals assayed.

Table 5. Inhibition by DPY-22 involves transcription factors other than BAR-1

Relevant genotype	% Vul*	% Muv†	VPC induction‡	*n*§	*P* value¶
dpy-22(sy622)	0	3	3.0±0.2	35	
bar-1(ga80)	55	0	2.3±0.8	20	
bar-1(mu63)	0	0	3.0±0.0	20	
pry-1(mu38)	26	22	2.9±0.5	23	
let-60(n1046)	0	73	4.0±0.8	22	
bar-1(mu63) dpy-22(sy622)	0	15	3.2±0.4	20	
bar-1(ga80) dpy-22(sy622)	55	0	2.3±0.9	22	
let-23(sy1)	100	0	0.5±0.9	24	
let-23(sy1); bar-1(mu63)	100	0	0.1±0.4	22	
pry-1(mu38); let-23(sy1)	8	84	4.0±0.9	25	<0.000001 versus *let-23(sy1)*
pry-1(mu38); let-23(sy1); bar-1(mu63)	52	30	2.7±0.9	27	0.00002 versus *pry-1(mu38); let-23(sy1)*
let-23(sy1); dpy-22(sy622)	75	0	2.1±0.9	20	<0.000001 versus *let-23(sy1)*
let-23(sy1); bar-1(mu63) dpy-22(sy622)	50	10	2.5±0.8	20	0.09 versus *let-23(sy1); dpy-22(sy622)*
let-60(n1046); bar-1(ga80)	0	3	3.0±0.1	29	
let-60(n1046); bar-1(ga80) dpy-22(sy622)	0	100	5.0±0.7	22	<0.000001 versus *let-60(n1046); bar-1(ga80)*
let-60(n1046); bar-1(ga80) dpy-22(sy622); syEx538 [F47A4]	0	15	3.1±0.3	54	0.05 versus *let-60(n1046); bar-1(ga80)* <0.000001 versus *let-60(n1046); bar-1(ga80) dpy-22(sy622)*

*Vulvaless. Percentage of animals that have less than three VPCs adopting vulval fates.
†Multivulva. Percentage of animals that have more than three VPCs adopting vulval fates.
‡Average number of VPCs that adopt vulval fates.
§Number of animals assayed.
¶*P* values were calculated for VPC induction using Student's *t*-test.

presence of only a weak vulval phenotype in *dpy-22(bx93)* mutants, suggest that the glutamine-rich region is dispensable for the function of DPY-22 in these processes. Because it is possible that small amounts of wild-type DPY-22 might be produced through translational readthrough in *dpy-22(bx93)* mutants, we directly examined the functional properties of a DPY-22 protein that completely lacks the glutamine-rich region. We injected a construct consisting of *dpy-22* DNA ending with the codon for amino acid 2548, fused in-frame to the GFP open reading frame, into *dpy-22(sy622)* animals (Fig. 2C). This transgene should produce a protein similar to that made in *dpy-22(bx93)* animals, which ends after amino acid 2548, before the beginning of the glutamine-rich region. Wild-type and glutamine-deleted DPY-22 fully rescued the body length, egg-laying and male tail defects observed in *dpy-22(sy622)* mutants (Fig. 1G,H; Table 2). Furthermore, glutamine-deleted DPY-22 also rescued the ectopic vulval fate specification observed in a *let-23(sa62)/+; dpy-22(sy622)* background, comparable with wild-type DPY-22 (Table 3). These results demonstrate that the glutamine-rich region is not essential for the majority of functions performed by DPY-22.

DISCUSSION

We have used a genetic approach to study mechanisms by which a diffusible growth factor can invariantly trigger a response in a particular cell within a field of competent cells. Initially using vulval tissue formation by male Pn.p cells as an assay, we identified two mutations in *dpy-22* that generally promote the activity of LET-23 in Pn.p cells, regardless of the sex of the animal. Our genetic analysis using the *dpy-22(sy622)*, *dpy-22(sy665)* and *dpy-22(bx93)* alleles, as well as RNAi against *dpy-22*, indicate that DPY-22 is normally an inhibitor of vulval fate specification (Tables 1, 3). As DPY-22 can act as an inhibitor in the absence of the LIN-3-producing anchor cell (Table 4), and DPY-22 is expressed in the vulval precursor cells during the time of vulval fate specification (Fig.

3B), we favor the idea that part of its function is executed in the VPCs. The ability of our *dpy-22* mutations to promote vulval fate specification in P5.p-P7.p, under conditions where RAS signaling is reduced (Tables 1, 4), indicates that DPY-22 acts as an inhibitor in all the VPCs, not just the VPCs that never adopt vulval fates (P3.p, P4.p and P8.p). The *dpy-22(sy622)* and *dpy-22(sy665)* mutations do not bypass the RAS pathway as they cannot fully suppress *lin-3* and *let-23* reduction-of-function mutations (Table 1). This suggests that DPY-22 acts directly on the RAS pathway or a pathway that cooperates with the RAS pathway and converges on a common target(s).

Besides the RAS pathway, LIN-12/NOTCH and WNT promote vulval fates. However, LIN-12 and RAS antagonize each other with respect to the type of vulval fate that is induced (Berset et al., 2001; Wang and Sternberg, 1999). High levels of LIN-12 signaling promote secondary vulval fates (Sternberg and Horvitz, 1989), and high levels of RAS signaling promote primary vulval fates (Katz et al., 1995). In our double mutants consisting of *dpy-22* and *let-23* pathway mutations, P6.p induction is restored (Table 1), and all animals displaying full vulval induction have correctly patterned vulvae consisting of 2°-1°-2° fates for P5.p-P7.p. These data indicate that *dpy-22* mutations cooperate with RAS to induce the primary fate in P6.p, rather than antagonize it, and suggest that DPY-22 does not act primarily by inhibiting the LIN-12 pathway.

DPY-22 has previously been described as an inhibitor of BAR-1-dependent WNT signaling (Zhang and Emmons, 2000). In contrast to the LIN-12-RAS relationship, RAS and WNT signaling are known to converge on at least one common target, necessary for vulval development, *lin-39* (Eisenmann et al., 1998; Maloof and Kenyon, 1998). Thus, DPY-22 inhibition likely occurs through interference with RAS and/or WNT signaling. However, our triple mutant analysis with a *bar-1* null mutation indicates that DPY-22 can inhibit activated RAS even in the absence of BAR-1 protein (Table 5). Together with our molecular data that the glutamine-rich region, which is necessary for inhibition of BAR-1-dependent ray development (Zhang and Emmons, 2000), is dispensable for inhibition of vulval development (Table 3), we propose that DPY-22 largely acts on the RAS pathway, rather than the WNT pathway, to inhibit vulval development. The strong gain-of-function *let-60(n1046)* allele only suppresses the *bar-1(ga80)* null mutation to wild type, and is itself suppressed for its multivulva phenotype by the *bar-1(ga80)* mutation (Table 5). Furthermore, our *dpy-22(sy622)* mutation which does not cause a strong multivulva phenotype, partially suppresses the moderate vulvaless phenotype of *let-23* pathway mutations (Table 1), but not the weak vulvaless phenotype of *bar-1(ga80)* (Table 5). These results suggest that shared targets between RAS and WNT signaling are unequally activated by the two pathways. In addition, it is likely that the WNT pathway has targets that are not shared with the RAS pathway.

Most inhibitors of vulval development affect all six VPCs. This observation is consistent with a model in which negative regulators of the RAS pathway raise the requirement for the amount of pathway activation needed to generate a biological response. Although the LIN-3 growth factor might diffuse outside of the source anchor cell, only P6.p, because of its proximity to the anchor cell, achieves sufficient pathway activation to adopt a primary vulval fate. A surprising number of points in the pathway must be negatively regulated, presumably to achieve invariant positional specificity for the response to LIN-3. SLI-1(c-CBL) (Jongeward et al., 1995; Yoon et al., 1995), GAP-1 (Hajnal et al., 1997) and LIP-1 (MAPK phosphatase) (Berset et al., 2001) appear to directly regulate LET-23 (EGFR), LET-60 (RAS) and SUR-1/MPK-1(MAP kinase), respectively, while ARK-1 might control some aspect of SEM-5(GRB2)-dependent RAS activation (Hopper et al., 2000).

Ultimately, the end point for growth factor signaling can be considered to be a change in RNA Pol II activity on specific promoters. Thus, in principle, sequence-specific and global regulators of transcription also might play important roles in regulating the output of the RAS pathway. Genetic studies have identified LIN-1, an Ets-domain sequence-specific DNA-binding protein as an inhibitor of vulval fate specification (Beitel et al., 1995). Global regulators of transcription broadly include chromatin remodeling proteins, RNA Pol II and the general transcription factors, and components of the mediator complex, which promote the activity of sequence-specific activators and repressors. Mutations in the synmuv genes *lin-35*(RB) and *lin-53*(RBAP48) have suggested that a histone deacetylase complex is inhibitory towards vulval fate specification (Lu and Horvitz, 1998). In support of this, experiments that directly examine HDAC-1 and components of the NURD complex have provided some evidence for their inhibitory roles (Chen and Han, 2001; Lu and Horvitz, 1998; Solari and Ahringer, 2000). Our work with *dpy-22*, which is most related to humanTRAP230 (Ito et al., 1999; Nagase et al., 1996; Philibert et al., 1998), now indicates that a specific component of the mediator also can act as an inhibitor of a RAS-dependent response.

At least 20 components of the mediator complex have been identified in yeast. Ten are essential for viability and seven of these appear to have related counterparts in higher species including *C. elegans* (for reviews, see Boube et al., 2002; Gustafsson and Samuelsson, 2001; Myers and Kornberg, 2000; Woychik and Hampsey, 2002). By contrast, the remaining yeast mediator components do not have clear orthologs in higher species. This has led to the proposal that these components and their counterparts in other species might not peform core mediator functions but, instead, specifically integrate information from rapidly evolving transcription factor families. Consistent with this model, mutation or RNA interference against the conserved components in *C. elegans* result in early lethality, or multiple phenotypes in the rare animals escaping lethality (Kwon et al., 1999; Kwon et al., 2001; Kwon and Lee, 2001). By contrast, the metazoan-specific mediator, *sur-2* (Boyer et al., 1999; Singh and Han, 1995), is not essential for viability, and single mutants have a strong vulvaless phenotype, resembling a RAS pathway mutation (Singh and Han, 1995). Similarly, prior to the discovery that *sop-1* was allelic to *dpy-22*, it was thought that mutations in *C. elegans* TRAP230, another metazoan-specific mediator component, do not affect viability and cause one major phenotype, which is the relief of inhibition on BAR-1-dependent regulation of *pal-1* expression in males (Zhang and Emmons, 2000).

However, our alleles of *dpy-22*, which cause more severe truncations of the protein and mislocalize it (Fig. 3I), result in multiple developmental and behavioral phenotypes (Fig. 1, Table 2) that do not appear to be restricted to one specific

signaling pathway. Some of these include an overall reduction in body length by 30%, a Dpy appearance, partial sterility (data not shown), abnormal ray development in the male tail and an egg-laying defect that does not result from the absence of vulval tissue or sex muscles, or a functional uterine-vulval connection (data not shown). We have also found that DPY-22 antagonizes vulval fates specified by activated RAS, and that the major target(s) of this inhibition is distinct from BAR-1 (Table 5). These findings are consistent with the original description of *dpy-22(e652)* phenotypes, and the observation that *dpy-22(e652)* enhances the multivulva phenotypes of *lin-53(n833); lin-15(n767)* and *lin-15(n765)* males (Meneely and Wood, 1987). Because we observe effects of *dpy-22* mutations on vulval development with multiple non-X-linked alleles in the RAS pathway, we do not think that DPY-22 acts on vulval development through regulation of dosage compensation from the X-chromosome, as originally proposed (Meneely and Wood, 1987). Neither loss-of-function nor gain-of-function mutants in the RAS pathway result in the body size or ray defects observed in our mutants, and *bar-1(mu63)* and *bar-1(ga80)* do not suppress at least the body size phenotype of our *dpy-22* mutants (Table 2, data not shown). These results suggest that transcription factors downstream of other signaling pathway also must be regulated by DPY-22. Similarly, recent data with *dpy-22(bx92)* and *sur-2* double mutants have shown a synthetic loss of rays (Zhang and Emmons, 2002), indicating SUR-2 also can function in pathways distinct from those using RAS and BAR-1. Thus, it is likely that DPY-22 and SUR-2 relay information from multiple activator/promoter contexts to RNA Pol II, but how they do it, and their relative contribution at a given promoter may be different.

The molecular identification of our new *dpy-22* alleles provides some insight into how a mediator component such as DPY-22 can have general and specific functions, depending on the promoter context. Although *dpy-22(bx93)*, which deletes the entire glutamine-rich domain of DPY-22, has a weak ability to promote ectopic vulval fate specification in the presence of activated LET-23, it has a profound ability to alleviate inhibition on BAR-1-dependent regulation of the *pal-1* gene (Zhang and Emmons, 2000). Furthermore, whereas a transgene harboring nonsense mutations affecting the glutamine-rich domain cannot restore inhibition to the *pal-1* gene as well as a wild-type *dpy-22* transgene (Zhang and Emmons, 2000), we find that a transgene completely lacking the glutamine-rich domain has the same activity as a wild-type transgene in rescuing vulval fate specification (Table 3), ray development (Fig. 1), body size and egg-laying defects (Table 2). The glutamine-rich region thus defines a domain that probably distinguishes promoter/activator-dependent activity for DPY-22. Inhibition of BAR-1-dependent *pal-1* expression is critically dependent on this domain, but not the activity of DPY-22 in the other pathways studied in this work. BLAST analysis (Boube et al., 2002; Gustafsson and Samuelsson, 2001) indicates the N-terminal region of TRAP230 is similar to both DPY-22 and SRB8 (Hengartner et al., 1995), a yeast mediator component without a clear ortholog in metazoans. SRB8 is responsible for recruiting (Myer and Young, 1998) the SRB10/SRB11 cyclin/cdk complex (Liao et al., 1995) that represses Pol II-dependent transcription on many promoters (Holstege et al., 1998). In cases where DPY-22/TRAP230 represses transcription, this function might be dependent on the SRB8-like domain, but modulated by the metazoan-specific domains. TRAP230 and PCQAP (Berti et al., 2001), two glutamine-rich components of human mediator complexes have been implicated in human disease. TRAP230 has been reported to undergo a 12 bp expansion in a region encoding the glutamine-rich domain in a subset of mentally retarded individuals (Philibert et al., 1998), and PCQAP is in the 22q11 deletion associated with DiGeorge syndrome (Berti et al., 2001). If TRAP230 and PCQAP contribute to these conditions, one would expect them to arise out of loss of specific and general functions, respectively.

Although compared with sequence-specific DNA-binding proteins, proteins involved in nucleosome displacement, histone acetylation and deacetylation, and components of the mediator complex appear to be global regulators of transcription, these global regulators may not act equally at all promoters. In particular, multiple chromatin remodeling and histone acetylation and deacetylation complexes with distinct subunit composition, substrate specificity and activator preferences have been described (reviewed by Emerson, 2002; Narlikar et al., 2002). Although it is still controversial whether distinct mediator complexes are associated with the RNA Pol II holoenzyme at a given promoter, mutations in different components have different effects on gene expression in yeast (Holstege et al., 1998; Myers et al., 1999). Some mediator components may perform unique functions for distinct activators or repressors, while others may be used to varying degrees or redundantly with other components. Finally, as in the case of DPY-22, a single component may use distinct domains to regulate transcription on different promoters. Genetic studies thus demonstrate that a specific component of the mediator provides an important point for regulating the output of the RAS pathway, and that specific mutations can be generated with distinct properties, which might contribute to disease in humans.

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