The *C. elegans* homolog of the mammalian tumor suppressor *Dep-1/Scc1* inhibits EGFR signaling to regulate binary cell fate decisions

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Protein phosphorylation by kinases and the subsequent dephosphorylation by phosphatases are key mechanisms that regulate intracellular signal transduction during development. Here, we report the identification of the receptor protein tyrosine phosphatase DEP-1 as a negative regulator of the *Caenorhabditis elegans* EGF receptor. DEP-1 amplifies in the developing vulva and the excretory system the small differences in the amount of EGF signal received by equivalent precursor cells to achieve binary cell fate decisions. During vulval development, DEP-1 inhibits EGFR signaling in the secondary cell lineage in parallel with the NOTCH-mediated lateral inhibition, while EGFR signaling simultaneously down-regulates DEP-1 and NOTCH expression in the primary cell lineage. This regulatory network of inhibitors results in the full activation of the EGFR/RAS/MAPK pathway in the primary vulval cells and at the same time keeps the EGFR/RAS/MAPK pathway inactive in the adjacent secondary cells. Mammalian *Dep-1/Scc1* functions as a tumor-suppressor gene in the intestinal epithelium. Thus, mutations in human *Dep-1* may promote tumor formation through a hyperactivation of the EGF receptor.

[Keywords: *C. elegans*; vulval development; EGFR; receptor protein tyrosine phosphatases; tumor suppressor]

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Several components of the EGF receptor signal transduction pathway are phosphorylated when an EGF ligand binds to its receptor (for review, see Schlessinger 2000). In particular, autophosphorylation of the EGFR at specific intracellular tyrosine residues is critical for the subsequent signal transduction. Protein phosphatases may be part of a negative regulatory network that attenuates the activity of the EGFR/RAS/MAPK pathway. The genomes of both invertebrates and vertebrates encode a large number of predicted phosphatase genes (Bhaduri and Sowdhamini 2003; Alonso et al. 2004). For example, the *Caenorhabditis elegans* genome contains at least 165 putative protein phosphatase genes (<http://www.wormbase.org>). The physiological substrates of most protein phosphatases have not yet been identified, as many experiments addressing this question were performed in vitro or by overexpression, which often impairs the substrate specificity of phosphatases (den Hertog 1999; Blanchetot et al. 2005). Only a few protein phosphatases have been assigned specific roles in developmental processes

or signaling pathways through clear-cut loss-of-function genetics. Examples include the mouse protein tyrosine phosphatase (PTP) 1B that inhibits insulin receptor signaling (Elchebly et al. 1999), *Drosophila* PTP-ER, which inhibits MAPK signaling during eye development (Karim and Rubin 1999) and *C. elegans* CLR-1, which inactivates the EGL-15 FGF receptor (Kokel et al. 1998). However, in many cases the identification and functional analysis of phosphatases is complicated by the fact that animals mutant for a single phosphatase gene display no obvious phenotype, suggesting that most protein phosphatases act redundantly (Harroch et al. 2000; Haj et al. 2003).

Mammalian *Dep1* is a member of the class III Receptor Protein Tyrosine Phosphatase (R-PTP) family (den Hertog 1999). *Dep-1* expression is induced in contact-inhibited cell cultures, hence the name Density-enhanced phosphatase 1 (Ostman et al. 1994). Different receptor tyrosine kinases (RTKs) including c-Met, PDGFR, and VEGFR-2 are *Dep-1* substrates in vitro, but whether these RTKs are also in vivo substrates of *Dep-1* is not known (Grazia Lampugnani et al. 2003; Jandt et al. 2003; Palka et al. 2003). Interestingly, *Dep-1* exhibits tumor-suppressor activity when overexpressed in cultured tu-

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mor cells (Keane et al. 1996; Trapasso et al. 2000), and the mouse *Dep-1* gene was recently identified as the colon cancer susceptibility locus *scc1* (Ruivenkamp et al. 2002). Human *Dep-1* is often mutated in colon, breast, skin, and lung carcinomas (Ruivenkamp et al. 2002). Despite its importance as a tumor suppressor in various epithelial tissues, the biological functions of *Dep-1* are not understood. Numerous questions remain to be answered to elucidate the role of *Dep-1* in tumorigenesis, including the identification of physiological substrates and the role of *Dep-1* in cell fate specification and pattern formation during normal development.

The development of the *C. elegans* hermaphrodite vulva serves as a paradigm to study how equivalent precursor cells can integrate the input from multiple signaling pathways to achieve a binary cell fate decision (Sundaram 2004). During vulval induction, the anchor cell (AC) in the somatic gonad secretes the EGF-like growth factor LIN-3 to activate the EGFR/RAS/MAPK pathway in the adjacent vulval precursor cells (VPCs). The strength of the EGFR/RAS/MAPK signal in the VPCs depends on their distance from the AC (Yoo et al. 2004). The VPC located closest to the AC, P6.p, exhibits highest RAS/MAPK activity and adopts the primary (1°) cell fate. The neighboring VPCs, P5.p and P7.p, exhibit intermediate levels of RAS/MAPK activity (Yoo et al. 2004), and the distal VPCs P3.p, P4.p, and P8.p that are further away from the AC display weak RAS/MAPK activity due to a relay signal generated by the proximal VPCs (Dutt et al. 2004). However, by the time of vulval cell fate specification at the beginning of the L3 stage, a lateral signal from P6.p that is transduced by the DELTA/NOTCH signaling pathway inactivates the EGFR/RAS/MAPK pathway in P5.p and P7.p to prevent 1° cell fate specification and induce the secondary (2°) fate in these cells (Ambros 1999; Berset et al. 2001; Chen and Greenwald 2004; Yoo et al. 2004). LIN-12 NOTCH signaling inhibits 1° fate specification in P5.p and P7.p by up-regulating the transcription of several negative regulators of the EGFR/RAS/MAPK signaling pathway such as *lip-1*, *dpy-23*, and the *lst* genes (Berset et al. 2001; Yoo et al. 2004). In particular, the dual-specificity phosphatase LIP-1, which is the homolog of vertebrate MKP-3, inactivates the MAP kinase MPK-1 to inhibit 1° fate specification in P5.p and P7.p (Berset et al. 2001). However, *lip-1(lf)* or *lst* RNA interference (RNAi) animals develop a morphologically wild-type vulva. Moreover, double mutants between *lip-1(lf)* and known inhibitors of the EGFR/RAS/MAPK signaling pathway such as *unc-101* (Lee et al. 1994), *sli-1* (Yoon et al. 1995), *gap-1* (Hajnal et al. 1997), or *ark-1* (Hopper et al. 2000) display no visible defects in lateral inhibition (T. Berset and A. Hajnal, unpubl.). These observations suggested that *lip-1* and the *lst* genes act redundantly with other inhibitors of the EGFR/RAS/MAPK pathway to achieve the binary, 1° versus 2° cell fate decision in the VPCs.

Here, we report the identification of the *C. elegans* R-PTP *dep-1* in a genetic screen for genes that regulate the 1° versus 2° cell fate decision during vulval development. DEP-1 negatively regulates LET-23 EGFR signal-

ing in the vulva and the excretory system in parallel with the MAPK phosphatases LIP-1 and independently of LIN-12 NOTCH. Similar to mutations in the mammalian *Dep-1/Scc1* tumor-suppressor gene, which alone are not sufficient to induce tumor formation (Ruivenkamp et al. 2002), the developmental phenotypes of *dep-1* only manifest in sensitized genetic backgrounds. Therefore, *C. elegans* vulval development serves as a genetic model to identify the homologs of mammalian tumor suppressor genes and study their genetic interactions with conserved signaling pathways.

Results

A genetic screen for mutants with defects in 2° cell fate specification

To identify new genes that regulate the 1° versus 2° vulval cell fate decision in parallel with *lip-1* (Berset et al. 2001), we screened ~30,000 haploid genomes in a *lip-1(lf)* background for mutants displaying defects in 2° cell fate specification using morphological criteria (Fig. 1A). Descendants of 1° vulval cells divide along the transverse (left-right) body axis, detach from the cuticle, and migrate inward (Katz et al. 1995). In this way, we isolated a loss-of-function mutation (*zh34*) in a previously uncharacterized gene that we named *dep-1* (see below). Besides the *zh34* mutation, we identified 15 mutations in other loci that cause a penetrant multivulva (Muv) phenotype but no apparent 2° cell fate transformation in P5.p or P7.p descendants (T. Berset and A. Hajnal, unpubl.). Lineage analysis of *dep-1(lf)*; *lip-1(lf)* double mutants and inspection of the vulval morphology in L4 larvae indicated that P5.p and P7.p descendants adopt a hybrid cell fate with 2° and 1° characteristics (a partial rotation of the cleavage planes in the last round of divisions followed by detachment from the cuticle), while *dep-1(lf)* or *lip-1(lf)* single mutants develop a wild-type vulva (Tables 1, 2; Fig. 1B–E). In addition, *dep-1(lf)*; *lip-1(lf)*

Table 1. *dep-1(lf)*; *lip-1(lf)* double mutants exhibit defects in the 2° cell lineage

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
Wild type	S or SS	S S	<u>LL</u> TN	TTTT	<u>NTLL</u>	S S
<i>lip-1(lf)</i>	S or SS	S S	<u>LL</u> TN	TTTT	<u>NTLL</u>	S S
<i>dep-1(lf)</i>	S or SS	S S	<u>LL</u> TN	TTTT	<u>NTLL</u>	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i>	S	S OO	OOTN	TTTT	NTOL	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i>	SS	S S	LOTN	TTTT	NTOL	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i>	S	S S	OOON	TTTT	<u>NTLL</u>	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i> ^a	SS	S S	LLON	TTTT	N?OL	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i>	SS	S S	OLTN	TTTT	<u>NTLL</u>	S S
<i>dep-1(lf)</i> ; <i>lip-1(lf)</i> ^a	SS	S S	LLL ^N	TTTT	NTOL	S S

Vulval cell divisions were followed by observation under Nomarski optics as described (Hajnal et al. 1997). The last round of cell divisions was scored as follows: (L) division along the anterior-posterior axis; (T) division along the left-right axis; (O) oblique cleavage plane; (N) refers to cells that did not divide during the third round of divisions. Cells that remained attached to the cuticle are underlined.

^aIn these two animals, the detachment could not be unambiguously scored. The alleles used were *dep-1(zh34)*, *lip-1(zh15)*.

Figure 1. Identification and morphological characterization of *dep-1*. (A) Genetic screen to identify mutants with 2° cell fate transformations. Wild-type vulva in an L4 larva (B) and adult (C). (D) Vulva of a *dep-1(lf); lip-1(lf)* L4 larva. Note that the P5.p and P7.p descendants have detached from the cuticula and moved inward (9). (E) Adult *dep-1(lf); lip-1(lf)* animal exhibiting a protrusion of vulval tissue due to the mixed 1°/2° cell fate of P5.p and P7.p descendants. (F,G) Overexpression of MPK-1 after the first round of VPC cell divisions causes a phenotype similar to the *dep-1(lf); lip-1(lf)* L4 larva shown in D. (H) *let-60(gf)* L4 larva. P5.p and P7.p have adopted the 2° fate, and their descendants remain attached to the cuticula, while some descendants of P4.p form an anterior pseudovulva (small arrow). (I) Adult *let-60(gf)* animal with a normal vulva in the center (large arrow) and an anterior pseudovulva (small arrow). A *dep-1(lf); let-60(gf)* L4 larva (J) and adult (K) animal exhibiting 2° cell fate transformations in addition to the Muv phenotype. (L) A *lin-12 notch(gf)* L4 larva, in which all VPCs have adopted the 2° fate. The orientations of the 2° fates are indicated with arrows. (M) A *dep-1(lf); lin-12(gf); lip-1(lf)* triple mutant displaying multiple 2° cell fate transformations. (N) Percentage of L3 larvae displaying *egl-17::cfp* expression in P5.px or P7.px cells. (O) Percentage of L3 larvae showing LET-23 expression in P5.p or P7.p descendants at the Pn.px and Pn.pxx stages. (P) LET-23 antibody staining of a *lip-1(lf)* and a *dep-1(lf); lip-1(lf)* mid-L3 larva at the Pn.pxx stage. MH27 staining of the adherens junctions in the animals is shown below. (Q) LIN-11::GFP expression from the *syIs80* transgene (Gupta et al. 2003) in P5.p and P7.p descendants of wild-type and *dep-1(lf); lip-1(lf)* L4 larvae at the Pn.pxxx stage. Arrows point at the LIN-11::GFP expressing vulval cells. Bars, 10 μm.

animals exhibit a weak Muv phenotype due to ectopic induction of P3.p, P4.p, or P8.p (Table 2). The same phenotype can be observed if the EGFR/RAS/MAPK pathway is hyperactivated after the first round of vulval cell divisions has occurred by overexpressing MPK-1 under the control of a heat-shock-inducible promoter (Fig. 1F,G; Table 2; Lackner and Kim 1998). Thus, prolonged MAPK signaling causes similar 2° cell fate defects as observed in *dep-1(lf); lip-1(lf)* double mutants, suggesting that *dep-1* is necessary to keep the EGFR/RAS/MAPK pathway inactive in the 2° cell lineage even after vulval induction. To further test whether *dep-1* inhibits 1° cell fate specification in the presumptive 2° cells, we removed *dep-1* function in sensitized genetic backgrounds exhibiting elevated EGFR/RAS/MAPK signaling. A gain-of-function mutation in the *let-60 ras* gene or a reduction-of-function mutation in *lin-15* causes a strong Muv phenotype, but loss of *dep-1* function additionally results in a transformation of the 2° cell fate in the P5.p and P7.p descendants similar to the defects observed in *dep-1(lf); lip-1(lf)* double mutants (Fig. 1H-K; Table 2).

Next, we used the *egl-17::cfp* reporter as a molecular

marker for the 1° cell fate (Burdine et al. 1998; Yoo et al. 2004). In a wild-type background, *dep-1(lf)* does not cause ectopic *egl-17::cfp* expression, but in a *let-60(gf)* background, *dep-1(lf)* increases the number of adjacent cells expressing *egl-17::cfp* (Fig. 1N). Loss of *dep-1* function does not significantly enhance *egl-17::cfp* expression in a *lip-1(lf)* background, probably because *lip-1(lf)* single mutants already exhibit frequent ectopic *egl-17::cfp* expression in P5.p and P7.p (Fig. 1N). Moreover, since *egl-17::cfp* expression does not always correlate with alterations in vulval morphology (Yoo et al. 2004), we tested LET-23 EGFR expression as another 1° cell fate marker (Kaech et al. 1998). In wild-type L3 larvae, LET-23 EGFR is strongly expressed in the 1° descendants of P6.p but is down-regulated in the 2° descendants of P5.p and P7.p. Ectopic LET-23 EGFR expression in 2° cells is rarely observed in *lip-1(lf)* or *dep-1(lf)* single mutants, but in 51% of *dep-1(lf); lip-1(lf)* double mutants, LET-23 EGFR persists in P5.p and/or P7.p descendants (Fig. 1O,P).

Finally, we tested whether *dep-1(lf)* affects the expression pattern of the 2° cell fate marker *lin-11::gfp* (Gupta et al. 2003). In wild-type mid-L4 larvae at the Pn.pxxx

Table 2. *DEP-1 inhibits LET-23 EGFR signaling*

Genotype	Induced VPCs/animal ^a	Vulval phenotype ^b	n
Wild type	3.0	wt	many
<i>lip-1(lf)</i>	3.0	wt	50
<i>dep-1(lf)</i>	3.0	wt	50
<i>dep-1(lf); lip-1(lf)</i>	3.2	75% 2° ⇒ 1°, 19% Muv	101
<i>lip-1(lf); gfp RNAi^c</i>	3.0	wt	20
<i>lip-1(lf); dep-1 RNAi^c</i>	3.1	53% 2° ⇒ 1°, 10% Muv	92
<i>dep-1(lf); lip-1(lf); zhEx90[dep-1(wt)]^d</i>	n.d.	3% 2° ⇒ 1°	33
<i>dep-1(lf); lip-1(lf); zhEx113[dep-1::gfp]^d</i>	n.d.	13% 2° ⇒ 1°	23
<i>let-60(gf)</i>	4.2	2% 2° ⇒ 1°, 70% Muv	43
<i>dep-1(lf); let-60(gf)</i>	5.5	30% 2° ⇒ 1°, 100% Muv	41
<i>lin-15(lf)</i>	6	49% 2° ⇒ 1°, 100% Muv	55
<i>dep-1(lf); lin-15(lf)</i>	6	97% 2° ⇒ 1°, 100% Muv	57
<i>hs::mpk-1</i> induced before VPC divisions ^c	5	100% 2° ⇒ 1°, 100% Muv	9
<i>hs::mpk-1</i> induced after VPC divisions ^c	3.4	50% 2° ⇒ 1°, 20% Muv	14
<i>lip-1(lf) gonad ablated</i>	0	100% Vul	18
<i>dep-1(lf) gonad ablated</i>	0	100% Vul	16
<i>dep-1(lf); lip-1(lf) gonad ablated</i>	1.0	4% 2° ⇒ 1°, 92% Vul	24
<i>let-23(rf); gfp RNAi^c</i>	0.5	96% Vul	25
<i>let-23(rf); dep-1 RNAi^c</i>	1.3*	77% Vul	31
<i>lin-2(lf)</i>	0.3	100% Vul	28
<i>dep-1(lf); lin-2(lf)</i>	2.3***	44% Vul	27
<i>lin-7(lf)</i>	0.2	100% Vul	24
<i>dep-1(lf); lin-7(lf)</i>	2.5***	20% Vul	18
<i>lin-10(lf)</i>	0.4	100% Vul	31
<i>lin-10(lf); dep-1(lf)</i>	1.3*	64% Vul	22
<i>sem-5(rf)</i>	0.9	76% Vul	25
<i>dep-1(lf); sem-5(rf)</i>	1.2	75% Vul	24
<i>bar-1(lf)</i>	2.5	30% Vul	43
<i>dep-1(lf); bar-1(lf)</i>	2.5	38% Vul	40

^aAverage number of VPCs per animal that have adopted a 1° or 2° vulval fate scored under Nomarski optics.

^b(% 2° ⇒ 1°) Fraction of animals, in which P5.p and/or P7.p have adopted a mixed 2°/1° fate as shown in Figure 1D and Table 1; (% Vul.) animals with fewer than three induced VPCs; (% Muv) animals with more than three induced VPCs; (***) a *p* value ≤ 0.0001; (**) a *p* value ≤ 0.001; (*) a *p* value ≤ 0.05.

^cRNAi experiments were done in the RNAi hypersensitive *rrf-3(lf)* background.

^dYoung adults were analyzed for their vulva morphology under a dissecting microscope. Three independent *dep-1(wt)* lines and one *dep-1::gfp* line were tested for rescue.

^eLarvae at the indicated stages were heat-shocked for 30 min at 33°C. See the Materials and Methods section for details on experimental procedures and alleles used.

stage, LIN-11::GFP is expressed in up to seven 2° descendants of each P5.p and P7.p (Fig. 1Q; on average, 4.6 cells per 2° lineage express LIN-11::GFP, *n* = 36). Expression in the distal-most VulA cells P5.paax and P7.pppx is usually weak or not detectable (Gupta et al. 2003). In contrast, in *dep-1(lf); lip-1(lf)* double mutants, LIN-11::GFP expression is absent or strongly reduced in most 2° cells (Fig. 1Q; on average, 1.8 cells per 2° lineage weakly express LIN-11::GFP, *n* = 60).

In summary, the morphological and molecular characterization of the cell fates in *dep-1(lf); lip-1(lf)* double mutants suggests that the *dep-1(lf)* phenotype is due to prolonged EGFR/RAS/MAPK signaling in P5.p and P7.p, which causes a transformation of the 2° cell fate into a fate with mixed 1° and 2° character.

dep-1 acts downstream of or in parallel with LIN-12 NOTCH

To examine the genetic interaction between *dep-1* and the *lin-12 notch* pathway, we tested whether *dep-1(lf)*

affects 2° fate specification in a *lin-12 notch(gf)* background, in which all six VPCs adopt the 2° cell fate (Greenwald et al. 1983). In *dep-1(lf); lin-12(gf)* or *lin-12(gf); lip-1(lf)* double mutants, all VPCs adopt the 2° fate (*n* = 23 and *n* = 22, respectively), but 74% of *dep-1(lf); lin-12(gf); lip-1(lf)* triple mutants showed a transformation of the 2° cell fate to a mixed 1°/2° fate (Fig. 1L,M; *n* = 23). Since *lin-12(gf)* animals have no AC, the somatic gonad in these animals produces no inductive LIN-3 EGF signal that could activate the LET-23 EGFR (Greenwald et al. 1983). However, gonad ablations demonstrated that vulval induction in *dep-1(lf); lip-1(lf)* animals can occur independently of the AC signal (Table 2). Thus, DEP-1 acts downstream of or in parallel with LIN-12 NOTCH to inhibit 1° fate specification.

dep-1 encodes a receptor protein tyrosine phosphatase

We identified F44G4.8 as the *dep-1* locus by positional cloning (Fig. 2A; Materials and Methods). *dep-1* encodes a protein of 1367 amino acids that exhibits strongest

Figure 2. Positional cloning of *dep-1*. (A) Map position, intron-exon structure, and fragments used for rescue and RNAi experiments. (B) Domain structure of DEP-1 (F44G4.8) compared with human DEP-1 and *Drosophila* Ptp4E. Open boxes represent the fibronectin-binding III repeats and black boxes the catalytic phosphatase domain. "I" indicates the percent sequence identity between the catalytic domains. The location of the *zh34* stop mutation and the catalytic center are highlighted. (C) Dendrogram showing the relationship between *C. elegans* (C.e.) DEP-1 (F44G4.8) and R-PTPs from *Drosophila melanogaster* (D.m.) and *Homo sapiens* (H.s.) calculated with the neighbor-joining method using the CLUSTAL X algorithm (Thompson et al. 1997). (D) Sequence alignment of the catalytic phosphatase domains of *C. elegans* DEP-1 with the catalytic domains of human class III R-PTPs. The asterisk indicates the position of the stop mutation in *dep-1(zh34)* animals, and the catalytic center containing the essential cysteine residue is underlined. GenBank accession numbers: (C.e.) DEP-1 CAA90125, (H.s.) DEP-1 Q12913, (H.s.) R-PTP-H NP002833, (H.s.) R-PTPβ P23467, (D.m.) PTP4E AAA76834, (D.m.) PTP10D P35992, (H.s.) R-PTP-RO AAH35960.

sequence similarity to the class III R-PTPs, which include the human Density-enhanced phosphatase-1 (DEP-1) (Ostman et al. 1994), R-PTP-H (Marneros et al. 2001), and R-PTPβ (Fig. 2B,C; Kaplan et al. 1990) as well as *Drosophila* Ptp4E and PTP10D (Yang et al. 1991). *dep-1* encodes the only *C. elegans* phosphatase that contains the domain arrangement characteristic of the class III R-PTPs, as defined by the presence of a single intracellular catalytic tyrosine phosphatase domain, a transmembrane domain, and multiple extracellular fibronectin type III repeats (den Hertog 1999). Since mammalian DEP-1 is the founding member of this R-PTP family, we named the *C. elegans* F44G4.8 gene *dep-1*.

C. elegans dep-1(zh34) animals carry a stop mutation at position 1162, truncating the protein before the catalytic center of the phosphatase domain (Fig. 2B,D). Since this mutation is predicted to destroy the catalytic phosphatase activity, *zh34* likely represents a loss- or strong reduction-of-function allele [*dep-1(lf)*]. Supporting these observations, RNAi against *dep-1* in a *lip-1(lf)* background induces a penetrant *dep-1* phenotype, and a genomic DNA fragment that spans the *dep-1* locus rescues the vulval defects of *dep-1(lf)*; *lip-1(lf)* animals (Fig. 2A; Table 2).

DEP-1 inhibits LET-23 EGFR signaling

Next, we tested whether *dep-1* negatively regulates the activity of the LET-23 EGFR during vulval induction.

dep-1(lf) suppresses the vulvaless (Vul) phenotype caused by *lin-7*, *lin-2*, or *lin-10* mutations, which reduce but do not eliminate the activity of *let-23 egfr* (Kaeck et al. 1998; Table 2). Furthermore, *dep-1* RNAi suppresses the *let-23 egfr(sy1)* reduction-of-function mutation. (Since *dep-1* maps within <0.1 map unit of *let-23*, we were unable to generate *dep-1*; *let-23* double mutants.) In contrast, a *sem-5 grb2(rf)* mutation, which blocks signal transduction downstream of *let-23 egfr* (Clark et al. 1992), is not suppressed by *dep-1(lf)* (Table 2). Since *wnt* signaling promotes vulval cell fate specification in parallel to the EGFR/RAS/MAPK pathway, we examined the interaction of *dep-1* with *wnt* pathway mutants (Gleason et al. 2002). A mutation in the β-catenin *bar-1* is not suppressed by *dep-1(lf)*, indicating that DEP-1 does not act downstream of BAR-1. Furthermore, a mutation in the negative regulator of the *wnt* pathway *pry-1 axin* (Korswagen et al. 2002) causes a Muv phenotype but no ectopic *egl-17::cfp* expression (Fig. 1N).

Confirming the results of the epistasis analysis, we observed a physical interaction between DEP-1 and LET-23 EGFR in GST pull-down experiments (Fig. 3). LET-23 EGFR from whole animal extracts specifically bound to the intracellular domain of DEP-1 carrying a substrate-trapping mutation in the phosphatase domain (GST::DEP-1D₁₂₄₁A) that allows the catalytically inactive enzyme to remain bound to its phosphorylated substrate (Palka et al. 2003). On the other hand, neither wild-type DEP-1 (GST::DEP-1wt) nor GST alone bound

Figure 3. A substrate trapping mutant of DEP-1 binds LET-23 EGFR. (*Left* panel) Binding of LET-23 EGFR from N2 worm lysates to GST::DEP-1 fusion proteins detected on a Western blot with polyclonal LET-23 antibodies. Twenty percent of the amount of N2 lysate used in the binding reactions was loaded in the *left-most* lane. The band around 150 kDa corresponds to full-length LET-23. “n.s.” indicates a nonspecific cross-reacting band since a similar-sized band was detected in extracts from mammalian cells lacking LET-23 expression. The same amounts of recombinant proteins used for the binding reactions were loaded on a parallel gel stained with Coomassie blue shown in the *right* panel. For details on the assay conditions, see Materials and Methods.

to LET-23 EGFR. Taken together, these results suggest that DEP-1 inhibits inductive AC signaling by dephosphorylating LET-23 EGFR.

DEP-1::GFP is expressed in 2° vulval cells independently of the lateral LIN-12 NOTCH signal

To investigate the *dep-1* expression pattern, we generated a transcriptional *dep-1p::gfp* and a rescuing translational DEP-1::GFP reporter line (Fig. 2A; Table 2; see also Materials and Methods). Both reporters are expressed at the time of vulval induction at equal levels in P5.p, P6.p, and P7.p (Fig. 4A). After the first round of cell divisions, the translational DEP-1::GFP reporter is down-regulated in the 1° lineage, but expression persists in the 2° vulval cells until the L4 stage (Fig. 4B–D; Supplementary Fig. S1). DEP-1::GFP expression is lower in P6.p than in P5.p or P7.p descendants in 82% of L3 and in 95% of L4 larvae ($n = 26$ and $n = 21$, respectively). The DEP-1::GFP expression pattern is summarized in Figure 4O. A similar reduction of the expression in the 1° lineage is observed with the transcriptional reporter, although the down-regulation in the 1° lineage only becomes apparent at the Pn.pxx stage (Fig. 4G; Supplementary Fig. S2). *dep-1p::gfp* expression is lower in P6.p than in P5.p or P7.p descendants in 80% of the L3 larvae ($n = 78$). In most cases (e.g., 17 out of 18 larvae at the Pn.px and Pn.pxx stages), DEP-1::GFP expression is low or absent in cells expressing high LET-23 EGFR levels and vice versa (Fig. 4E). Most of the DEP-1::GFP fusion

protein is localized in intracellular punctae that partially colocalize with intracellular LET-23 EGFR (Fig. 4L,M).

Since the DEP-1::GFP expression pattern is very similar to that of a translational LIN-12::GFP reporter, we tested if *dep-1* transcription is controlled by LIN-12 NOTCH (Levitan and Greenwald 1998; Shaye and Greenwald 2002). Although the *dep-1p::gfp* transcriptional reporter is expressed in ectopic 2° cells of *lin-12(gf)* mutants, no obvious up-regulation can be observed when compared with *dep-1p::gfp* levels in the 2° VPCs of wild-type animals (Fig. 4G,H; Supplementary Fig. S2). Unlike direct LIN-12 NOTCH target genes such as *lip-1* (Berset et al. 2001), the P5.p and P7.p descendants continue to express *dep-1p::gfp* in a *lin-12 notch(lf)* background in 89% of the cases, although expression levels are slightly reduced in the P5.p and P7.p descendants ($n = 37$) (Fig. 4I; Supplementary Fig. S2). Furthermore, no

Figure 4. DEP-1::GFP expression pattern during vulval development. (A–D) Expression pattern of the translational DEP-1::GFP reporter during vulval development. Note in C the lower levels of DEP::GFP in the two distal cells of the 2° lineages at the Pn.pxx stage. (E) Mutually exclusive DEP-1::GFP and LET-23 antibody staining at the Pn.pxx stage. (F) EGL-17::YFP expression at the Pn.pxx stage. Expression of the transcriptional *dep-1p::gfp* reporter in wild-type (G), *lin-12(gf)* (H), and *lin-12(lf)* (I) larvae at the Pn.pxx and Pn.px stages, respectively. Persisting DEP-1::GFP expression in all (J) or half (K) of the induced cells in *sur-2(lf)* mutants. Subcellular localization of DEP-1::GFP (L) and LET-23 (M) at the Pn.px stage. (N) Merged image. Arrows point at intracellular punctae containing both DEP-1::GFP and LET-23. Bars: N, 5 μ m; otherwise, 10 μ m. (O) Summary of the observed DEP-1::GFP expression pattern.

Table 3. *DEP-1 acts in the 2° cell lineage*

Row	P5.p	P6.p	P7.p	Probable loss of array in ^a	<i>n</i>
1	2° +	1° +	2° +	no loss	486 ^b
2	2° ⇒ 1° -	1° -	2° ⇒ 1° -	AB.p	7
3	2° ⇒ 1° -	1° +	2° -	AB.pl & >AB.pl ^c	2
4	2° ⇒ 1° -	1° +	2° ⇒ 1° -	AB.pr	1
5	2° ⇒ 1° -	1° +	2° +	AB.pl	1
6	2° +	1° -	2° ⇒ 1° -	AB.pr	1
7	2° +	1° -	2° +	AB.pl, AB.pr & >AB.pr ^d	3

^aSee Materials and Methods for details on the identification and scoring of mosaic animals. (+) Presence of the rescuing array in the descendants of the respective VPC; (-) absence of the array; (2° ⇒ 1°) a transformation of the 2° fate to a mixed 1°/2° fate as described above (Figs. 1D, 5A; Table 1). The genotypes of P3.p, P4.p, and P8.p descendants could not be reliably scored because at the L4 stage they had fused with hyp7 and hence expressed GFP irrespective of the presence of the rescuing array.

^bOne of the 486 animals exhibited the mutant phenotype despite the presence of the rescuing array in all vulval cells.

^{c,d}In these two animals, the array had been lost in those parts of the AB.pl and AB.pr lineage, respectively, that gave rise to the P cells since both vulval cells and Pn.a-derived neurons lacked GFP expression.

cluster of CSL sites characteristic of the known LIN-12 NOTCH targets can be found in the regulatory region of *dep-1* (Berset et al. 2001; Yoo et al. 2004). Thus, *dep-1* is probably not a direct transcriptional target of the lateral DELTA/NOTCH signaling pathway.

EGFR signaling coordinately down-regulates DEP-1 and LIN-12 NOTCH expression in the 1° vulval cells

In *lin-12(gf)* animals, which lack an AC and hence produce no inductive signal (Greenwald et al. 1983), *dep-1p::gfp* remains expressed in the P6.p descendants (Fig. 4H), suggesting that EGFR/RAS/MAPK signaling down-regulates *dep-1* expression in 1° cells. Supporting this idea, we observed an asymmetric DEP-1::GFP expression pattern among the 2° vulval cells that is complementary to the pattern of the EGFR/RAS/MAPK target *egl-17* (Burdine et al. 1998; Cui and Han 2003). DEP-1::GFP levels are consistently higher in the proximal 2° cells (P5.ppx and P7.pax) that are in direct contact with the 1° descendants of P6.p that produce the lateral signal (Fig. 4C,O). Conversely, EGL-17::YFP expression is higher in the distal 2° cells (P5.pax and P7.ppx) that probably receive less lateral signal and hence exhibit higher EGFR/RAS/MAPK pathway activity (Fig. 4F). To test whether EGFR/RAS/MAPK signaling represses DEP-1 transcription, we examined the DEP-1::GFP expression pattern in *sur-2(lf)* animals. *sur-2* encodes a component of the mediator complex that acts downstream of the

EGFR/RAS/MAPK pathway to induce LIN-12 NOTCH down-regulation in 1° cells and activate lateral signaling (Singh and Han 1995; Shaye and Greenwald 2002). In *sur-2(lf)* mutants, a 1° vulval lineage is frequently induced in P6.p, but no 2° cells are specified because P6.p produces no DSL ligands (Chen and Greenwald 2004). Similar to LIN-12::GFP, DEP-1::GFP remains expressed in *sur-2(lf)* mutants in all or half of the cells in the 1° lineage (Fig. 4J,K). Eighty percent of *sur-2(lf)* animals express DEP-1::GFP in the 1° lineage compared with 9% in a wild-type background, *n* = 54 and 34, respectively.

Thus, the AC signal simultaneously down-regulates DEP-1 and LIN-12 NOTCH expression in the 1° vulval cells via the SUR-2 transcription factor.

DEP-1 represses EGFR signaling in the 2° cell lineage

Although DEP-1::GFP is expressed at highest levels in the 2° cells, the defects in 2° cell fate specification could be an indirect consequence of a loss of *dep-1* function in the 1° cell lineage. To distinguish between a cell-autonomous function of *dep-1* in 2° cells and a cell-non-autonomous function in the 1° cells, we performed a *dep-1* mosaic analysis in a *lip-1(lf)* background. We identified 15 mosaic animals that fall into three classes (Table 3). (1) Seven animals were mutant for *dep-1* in all vulval cells. In all of these cases, the P5.p and P7.p descendants exhibited a transformation of the 2° cell fate to a mixed 1°/2° cell fate as described above (Table 3, row 2). (2) Four

Figure 5. Cell-autonomous function of DEP-1 in the 2° cell lineage. Two examples of the informative mosaic animals. Arrows indicate the position of the nuclei of P5.p and P7.p descendants; arrowheads point at the nuclei of P6.p descendants. (A,C) Morphology of the P5.p and P7.p descendants at the L4 stage. (B,D) Expression of the SUR-5::GFP cell lineage marker indicating presence of the rescuing *dep-1(+)* array *zhEx90*. The insets show the upper focal planes where four of the eight P6.p descendants are located. (A,B) The animal from row 5 in Table 3. The descendants of P5.p lacked *dep-1*, while P6.p and P7.p descendants were wild type, resulting in a cell fate transformation of the P5.p descendants. (C,D) One of the three animals from row 7 in Table 3 in which the descendants of P6.p lacked *dep-1*, while P5.p and P7.p descendants were wild type. P5.p and P7.p adopted a normal 2° fate in this animal. Bar: D, 10 μm.

Figure 6. DEP-1 controls duct cell specification. (A) Lineage relationship between the duct cell precursors and phenotypes caused by *let-60 ras* mutations (Yochem et al. 1997). Nomarski (B) and GFP fluorescence (C) image of the duct cell in a wild-type L3 larva carrying the LIN-48::GFP reporter (Sewell et al. 2003). (D,E) *let-60(gf)* larva containing two duct cells. (F,G) *dep-1(lf); lip-1(lf)* animal exhibiting duct cell duplication. Arrows point at the nuclei of LIN-48::GFP-expressing cells. Bar, 5 μ m. (H) Quantification of the duct cell duplications. "n" indicates the number of larvae scored for each genotype.

animals lacked *dep-1* in P5.p and/or P7.p descendants, while the P6.p descendants contained the *dep-1* transgene (Table 3, rows 3–5; Fig. 5A,B). In six out of eight cases, in which P5.p or P7.p was mutant for *dep-1*, we observed a 2° cell fate transformation (including the animal from row 6 in Table 3). (3) Three animals expressed *dep-1* in the P5.p and P7.p descendants but not in the P6.p descendants (Table 3, row 7; Fig. 5C,D). In these mosaic animals, P5.p and P7.p generated a normal 2° cell lineage and a wild-type vulva developed. Taken together, the mosaic analysis points at a cell-autonomous function of *dep-1* in the 2° cell lineage. These findings further support the idea that the 2° cell fate transformation is caused by elevated LET-23 EGFR signaling in the 2° cell lineage.

DEP-1 regulates the binary cell fate choice in the excretory system

To examine another example of a binary cell fate decision controlled by the EGFR/RAS/MAPK pathway, we asked whether DEP-1 regulates the specification of the duct cell fate in the excretory system (Fig. 6A; Yochem et al. 1997). During embryonic development, one descendant of each of the AB.pl and the AB.pr lineage has the potential to adopt the duct cell fate (Sulston et al. 1983). In wild-type animals, the AB.pl descendant differentiates into the duct cell, while the AB.pr descendant adopts an alternative cell fate, which presumably generates the G1

cell that forms the excretory pore in the embryo and a neuroblast in the larva (Sulston et al. 1983). *let-60 ras* gain-of-function mutants develop two duct cells, whereas *let-60 ras* loss-of-function mutants contain no duct cell (Fig. 6A–E; Yochem et al. 1997). If the EGFR/RAS/MAPK pathway is inactivated in the AB.pl lineage or the duct cell precursor is ablated, then the precursor cell from the AB.pr lineage generates the duct cell, indicating that the AB.pl-derived duct cell or its precursor normally inhibits the equipotent cell in the AB.pr lineage from producing a duct cell (Sulston et al. 1983; Yochem et al. 1997). *dep-1(lf); lip-1(lf)* double mutants frequently display duct cell duplications as shown by the ectopic expression of the *lin-48::gfp* marker (Fig. 6F–H; Sewell et al. 2003). In contrast, *pry-1 axin(lf)*, *bar-1(gf)*, and *lin-12 notch(lf)* mutants never have more than one duct cell, and *lin-12(gf)*, *glp-1 notch(gf)*, and *bar-1(lf)* mutants always contain a duct cell (Fig. 6H; Lambie and Kimble 1991). Thus, neither the *wnt* nor the *lin-12 notch* pathways control duct cell fate specification. Therefore, *dep-1* inhibits EGFR/RAS/MAPK signaling in the duct cell precursors independently of the *wnt* and *notch* signals.

Discussion

DEP-1 specifically inhibits LET-23 EGFR signaling

We have identified the R-PTP DEP-1 in a forward genetic screen for genes that regulate the 1° versus 2° cell fate

Figure 7. A model for DEP-1 function during vulval development. The inductive AC signal simultaneously down-regulates LIN-12 NOTCH and DEP-1 expression in P6.p via SUR-2, while LIN-12 and DEP-1 block the transduction of the inductive AC signal in P5.p and P7.p.

choice decision during *C. elegans* vulval development. Several lines of evidence indicate that DEP-1 specifically inhibits the transduction of the AC signal by dephosphorylating the EGFR homolog LET-23. First, LET-23 EGFR is the only RTK known to regulate 1° vulval fate as well as duct cell specification. In both of these tissues, *dep-1(lf)* leads to the same phenotype as increased EGFR/RAS/MAPK signaling. Second, the genetic epistasis analysis indicates that DEP-1 inhibits 1° fate specification upstream of the adaptor protein SEM-5 GRB2. In addition, *dep-1(lf)* in combination with *lip-1(lf)* renders vulval induction partially independent from the AC signal, suggesting that in a *dep-1(lf)* background the AC-independent activity of LIN-3 EGF (Dutt et al. 2004) becomes sufficient to induce vulval differentiation. Third, protein interaction experiments show that the intracellular domain of DEP-1 is sufficient to bind to LET-23, as a substrate-trapping mutant of DEP-1 but not wild-type DEP-1 captures LET-23 EGFR from total animal lysates. This observation indicates that DEP-1 specifically binds to phosphorylated LET-23 via its substrate-binding pocket in the phosphatase domain (Palka et al. 2003). Fourth, DEP-1::GFP and LET-23 EGFR are colocalized in intracellular punctae, suggesting that DEP-1 dephosphorylates LET-23 after the receptor has undergone ligand-induced endocytosis. Similarly, mammalian PTP-1B interacts with internalized PDGFR and EGFR on the surface of the endoplasmic reticulum (Haj et al. 2002).

On the other hand, we did not observe a function of DEP-1 in developmental processes controlled by other RTKs. For example, *dep-1(lf)* suppresses neither the Dauer phenotype caused by a mutation in the *daf-2* insulin receptor nor the EGL phenotype caused by an *rf* mutation in the *egl-15* fibroblast growth factor receptor (Kokel et al. 1998; data not shown). Also, the polarity reversal of the 2° cell fate in P7.p caused by a mutation in the *lin-17* frizzled receptor that acts together with the *lin-18* Ryk/derailed family RTK is not affected by *dep-1(lf)* (data not shown). On the other hand, RNAi analysis of 121 of the 165 predicted *C. elegans* protein phosphatase genes in a *lip-1(lf)*

and *dep-1(lf)* background has identified no additional phosphatases that show a genetic interaction with *lip-1* or *dep-1* (U. Forster and A. Hajnal, unpubl.). Taken together, these observations point at a relatively high specificity of DEP-1 for LET-23 EGFR. This specificity may be achieved through a combination of the intrinsic substrate specificity of the phosphatase domain with a tightly regulated and dynamic expression pattern that maintains DEP-1 expression in a subset of cells and a restricted subcellular localization that allows DEP-1 to act only on internalized LET-23 EGFR.

DEP-1 inhibits 1° cell fate specification in parallel with the LIN-12 NOTCH pathway

Lateral inhibition mediated by the NOTCH signaling pathway controls binary cell fate decisions during the development of most if not all animals (for review, see Lai 2004). In the developing vulva, LIN-12 NOTCH was found to inhibit 1° cell fate specification in the future 2° cells by inducing the transcription of a set of inhibitors of the inductive EGFR/RAS/MAPK pathway (Berset et al. 2001; Yoo et al. 2004). Although DEP-1::GFP expression is highest in 2° vulval cells, *dep-1* is not a direct LIN-12 NOTCH target gene. Rather, DEP-1 and LIN-12 NOTCH expression is simultaneously repressed in P6.p by the inductive EGFR/RAS/MAPK signaling pathway, which creates a positive feedback loop in the 1° cell lineage (Fig. 7). Similar to LIN-12 NOTCH, the down-regulation of DEP-1 is mediated by the SUR-2 transcription factor (Shaye and Greenwald 2002). Since LIN-12 NOTCH signaling inactivates the EGFR/RAS/MAPK pathway in P5.p and P7.p, DEP-1 expression persists in the 2° cells. Thus, DEP-1 inhibits LET-23 EGFR signaling in parallel with LIN-12 NOTCH to ensure a stable inhibition of the 1° fate in P5.p and P7.p and their descendants. This conclusion is further supported by the finding that *dep-1* controls the duct cell fate decision in the excretory system independently of the NOTCH pathway.

Why is such a complex regulatory network with multiple, genetically redundant inhibitors required for vulval pattern formation? Beginning in the L2 stage, the AC generates a gradient of LIN-3 EGF, which causes the activation of the EGFR/RAS/MAPK pathway in the proximal VPCs (P5.p, P6.p, and P7.p) (Yoo et al. 2004). Furthermore, a relay signal from the proximal VPCs propagates the AC signal to the distal VPCs (P3.p, P4.p, and P8.p) (Dutt et al. 2004). Even after vulval induction, the AC continues to express LIN-3 throughout the following vulval cell divisions. One explanation is therefore that the NOTCH-mediated lateral inhibition, which begins in the G1 phase of the VPC cell cycle only after the AC and relay signals have been received by the VPCs, does not irreversibly inhibit 1° cell fate specification in P5.p and P7.p (Ambros 1999). Indeed, a 2° cell can still be converted into a 1° cell by increasing the MAPK signal until the G1 phase of the following cell cycle (Wang and Sternberg 1999). Thus, the repression of the EGFR/RAS/MAPK pathway in the 2° cells must be maintained for at

least one more round of cell divisions. DEP-1 expression in the 2° vulval cell lineage may be necessary to sustain the effect of lateral inhibition until the daughter cells of P5.p and P7.p have irreversibly adopted the 2° fate.

Another possible answer lies in the developmental flexibility of vulval development. For example, if P6.p lacks LET-23 EGFR, then P5.p will adopt the 1° instead of the 2° fate it normally adopts, and its neighbors P4.p and P6.p will differentiate into 2° cells instead of adopting the 3° and 1° fate, respectively (Simske and Kim 1995). Developmental flexibility may become even more important in higher organisms, where individual cells must be selected from much larger groups of precursor cells. It will therefore be interesting to see if DEP-1 performs a similar role during the development of higher organisms.

C. elegans as a model to study tumor-suppressor gene function

Our results establish *C. elegans* as a model to study the function of *dep-1* during development, which may lead to an understanding of the molecular mechanism underlying the tumor suppressor activity of mammalian *Dep-1*. Loss of *C. elegans dep-1* function alone does not cause a visible developmental defect, but it shifts the balance in the uncommitted VPCs from the 2° toward the 1° cell fate. Similarly, mutations in human *Dep-1*/*Sccl* alone are not sufficient to induce oncogenic transformation, but they increase the likelihood of tumor development in various epithelial tissues, especially in the intestinal mucosa (Ruivenkamp et al. 2002). In analogy to the function of *C. elegans* DEP-1, the defect leading to the formation of intestinal carcinomas may be a failure to restrict EGFR-stimulated cell proliferation in the epithelia as EGFR expression or activity is up-regulated in many human carcinomas (Holbro and Hynes 2004). At the base of intestinal crypts, stem cells can either proliferate or differentiate into migratory enterocytes that leave the base of the crypts. Possibly, loss of DEP-1 function shifts this balance, leading to an accumulation of proliferating undifferentiated cells that may further develop into tumor cells.

Materials and methods

General *C. elegans* methods and strains

C. elegans strains were cultured at 20°C as described (Brenner 1974). Wild type refers to *C. elegans* variety Bristol, strain N2. The mutations used are listed below. Unless noted otherwise, the mutations used have been described (Hodgkin 1997). Standard methods were used to construct double and triple mutants. Unless noted in the table legend, all experiments were conducted at 20°C. *dep-1(zh34)* was *cis*-linked with *unc-4(e120)* or *bli-1(e769)* to construct the double mutants shown in Tables 1–3. The induction of VPCs was scored at the L4 larval stage using Nomarski optics as described (Berset et al. 2001). To ablate the gonad, the nuclei of the Z1–Z4 gonad precursor cells were ablated in early L1 larvae with a laser microbeam as de-

scribed (Kimble 1981). The operated animals were allowed to develop until the L4 stage, when successfully ablated animals, in which neither gonad arm had developed and no residual gonadal cells survived, were identified under Nomarski optics.

The alleles used are LGI: *lin-10(e1438)*, *unc-13(e1091)*, *unc-101(sy108)*, *sur-2(ku9)*, *pry-1(mu38)*; LGII: *dep-1(zh34)* (this study), *lin-7(e1413)*, *unc-4(e120)*, *dpy-10(e128)*, *bli-1(e769)*, *let-23(sy1)*, *rrf-3(pk1426)* (Simmer et al. 2002). LGIII: *unc-119(e2498)*, *dpy-19(e1259)*, *lin-12(n137n720lf)*, *unc-32(e189)*, *lin-12(n137gf)*, *daf-2(e1370)*. LGIV: *lip-1(zh15)* (Berset et al. 2001), *let-60(n1046gf)*, *lin-3(e1417)*, *unc-5(e53)*, *ark-1(sy247)* (Hopper et al. 2000); LGX: *shl-1(sy143)* (Jongeward et al. 1995), *gap-1(ga133)* (Hajnal et al. 1997), *lin-15(n309)*, *sem-5(n2019)*, *lin-2(n397)*, *bar-1(ga80)* (Eisenmann et al. 1998).

The transgenic arrays (transgene, cotransformation marker) were *sals14[lin-48::gfp, unc-119(+)]* (Sewell et al. 2003), *arIs92[egl-17p::cfp-lacZ,unc-4(+)]* (Yoo et al. 2004), *gaIs36[HS-mpk-1(+),EF1alpha-D-mek(+),unc-30(+)]* V (Lackner and Kim 1998), *zhIs10[dep-1p-Δpes::nls::gfp::lacZ, unc-119(+)]* (this study), *syIs80[lin-11::gfp, unc-119(+)]* (Gupta et al. 2003), *zhEx112[dep-1::gfp, unc-119(+)]* (this study), *zhEx113[dep-1::gfp, lin-48::gfp]* (Johnson et al. 2001), *zhEx90[F44G4.8, sur-5::gfp]* (this study).

Positional cloning of *dep-1*

Young adult *lip-1(zh15)* hermaphrodites were mutagenized with 50 mM ethyl methanesulfonate for 4 h at room temperature, and the F2 generation was screened for mutants displaying 2° cell fate defects. Approximately 30,000 haploid genomes were screened. *dep-1(zh34)*; *lip-1(zh15)* mutants were backcrossed three times against Bristol N2, and chromosomal linkage of *zh34* to LGII was determined by bulk-segregant analysis as described (Wicks et al. 2001). Three factor mapping placed *dep-1(zh34)* between *dpy-10* and *unc-4*, approximately at position 0.9. For high-resolution SNP mapping, *unc-4(e120)* *dep-1(zh34)*; *lip-1(zh15)* and *dpy-10(e128)* *dep-1(zh34)*; *lip-1(zh15)* animals were crossed with the polymorphic mapping strain *C. elegans* CB4856 (Wicks et al. 2001). Pvl nonUnc and Pvl nonDpy recombinants were isolated, and F3 clones homozygous for the recombinant chromosome were established. Informative SNPs were selected from the *C. elegans* Genome Sequencing Center Web site (<http://genome.wustl.edu>). SNP analysis defined a region between snp_F37B12 and snp_T07D4 (Fig. 2A). RNAi analysis (see below) of 54 of the predicted genes in this region in an *rrf-3(pk1426)*; *lip-1(zh15)* background identified F44G4.8 as the candidate gene. Rescue experiments were performed as follows: 20.6-kb PCR products were amplified with TaKaRa LA Taq (TAKARA BIO INC) using the primers OTB57 (GGATTTTGAACCATTTGGGTCTG) and OTB44 (ATGAGCTACCCGTGGAAACC) from the cosmid F44G4, pooled, and tested for integrity by restriction enzyme digestion. Thirty nanograms per microliter of purified PCR product was injected together with 50 ng/μL of pTG96 (*sur-5::gfp*) as a transformation marker into wild-type animals, and three independent transgenic lines were crossed to *dep-1(zh34)*; *lip-1(zh15)* mutants to assay for rescue.

GFP reporter constructs

For the *dep-1p::gfp* transcriptional reporter, a 6.1-kb fragment from the 5'-region of *dep-1* was PCR-amplified from wild-type genomic DNA with the primers OTB77 (GCATGCCGAAAGT GATCGAATTGACCG) and OTB76 (GCATGCGTAGTTTT TCCGCAACTCGTG), digested with SphI, and cloned into pTB11. pTB11 contains a 370-bp BamHI fragment with a *Δpes*-

10 minimal promoter isolated from pPD95.21 and inserted into the BamHI site of the *nls::gfp::lacZ* reporter plasmid pPD96.04. (All pPD plasmids are gifts of A. Fire, Stanford, CA.) The translational *dep-1::gfp* reporter was constructed as follows: A 5.6-kb genomic PCR fragment that includes 4.5 kb of the *dep-1* upstream region was amplified from wild-type DNA with the primers OTB94 (GTTCGACGGATTTTGAACCATTGGGTCTG) and OTB70 (GGTCTACTACTCATTCCGTC), digested with SalI and BamHI and cloned into pPD95.75. A second fragment of 3.7 kb was amplified from *dep-1* cDNA with the primers OTB93 (TTTGCCATCAAGAAGGATTCC) and OTB92 (GGATCCC GAAATCCCGATTGACAATCAT), digested with BamHI, and cloned in frame to *gfp*.

Transgenic animals

Transgenic animals were generated either by microinjection of DNA or by microparticle bombardment as described (Mello et al. 1991; Praitis et al. 2001). Where indicated, extrachromosomal arrays were integrated into the genome by γ -irradiation of L4 larvae. Integrated arrays were backcrossed at least five times before further analysis.

GST pull-down experiments

A cDNA fragment encoding the intracellular domain of DEP-1 (amino acids 925–1367) was cloned into the BamHI site of the *Escherichia coli* expression vector pGEX-2TK (Pharmacia). The D₁₂₄₁-to-A mutation was introduced by site-directed mutagenesis using the Quick-Change Kit (Stratagene). Recombinant proteins were affinity-purified on glutathione Sepharose beads according to the manufacturer's protocol (Pharmacia), except that protein expression was induced in BL21 bacteria at 25°C, and fusion proteins were extracted in the presence of 0.1% Triton X-100 and 450 mM NaCl. Approximately 1 μ g of each DEP-1 fusion protein and 10 μ g of GST as negative control were used in each binding reaction. To prepare N2 worm extracts, mixed-stage cultures were washed three times with PBS, resuspended in lysis buffer (25 mM Tris at pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 1 $\times$ protease inhibitor cocktail; Roche), homogenized with a douncer, and centrifuged for 15 min at 4°C and 10,000g to remove insoluble components. About 100 μ g of total protein extract was used in each reaction. Binding was performed at room temperature for 2 h, the Sepharose beads were washed three times with lysis buffer, and bound proteins were eluted by boiling the beads for 5 min in SDS gel-loading buffer. LET-23 was detected on Western blots of 10% acrylamide gels with polyclonal affinity-purified LET-23 antibody (Kaech et al. 1998). Similar results were obtained using a monoclonal antibody raised against the same LET-23 epitope (data not shown).

Antibody staining and GFP expression studies

LET-23 antibody staining using a polyclonal antibody against the LET-23 C terminus was performed as described in Kaech et al. (1998). GFP and CFP expression was observed under fluorescent light illumination with a Leica DMRA microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software package (Improvision). To score GFP expression in the Pn.p cells, animals were mounted on 3% agarose pads in M9 solution containing 15 mM NaN₃. Larvae were first inspected using Nomarski optics to identify the position of the Pn.p cells or their descendants, and GFP expression was then scored under fluorescent light illumination using the

same exposure settings for a particular transgene in all different genetic backgrounds.

RNAi interference

RNAi was performed by feeding worms with dsRNA-producing *E. coli* as described (Kamath et al. 2001) with the following modifications: Five to 10 L1 larvae were placed on the RNAi plates, and the P0 and F1 generations were inspected for vulval phenotypes. Three millimolar IPTG was added to the agar to induce the expression of dsRNA.

Mosaic analysis

Mosaic analysis was done as described (Hajnal et al. 1997) except that *sur-5::gfp* was used as a cell lineage marker instead of *ncl-1*. The *sur-5::gfp* marker has the advantage over *ncl-1* that the genotype of the vulval cells can be reliably scored under fluorescence light illumination (Yochem et al. 1997). L4 larvae of the genotype *dep-1(zh34) unc-4(e120); lip-1(zh15); zhEx90.3[F44G4.8, sur-5::gfp]* were anesthetized with 10 mM NaN₃ and inspected under Nomarski optics in batches of 20–30 animals. First, animals exhibiting a mutant vulval phenotype were scored for GFP expression in the following cells to infer the most likely site of loss of the rescuing array: intestinal cells, anchor cell, body wall muscles in the head and distal tip cells (all EMS derived), ASKR, ADLR head neurons, excretory cell, Pn.a-derived motor neurons and the vulval cells (all from the AB.p lineage), and ASKL and MI (AB.a-derived head neurons). Finally, animals exhibiting a wild-type vulval phenotype were screened for a loss of the rescuing array in the 1° or 2° vulval cells to identify those mosaics that permit wild-type vulval development (Table 3, row 7).

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