Development 125, 181-190 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV9546

The Hox gene *lin-39* is required during *C. elegans* vulval induction to select the outcome of Ras signaling

Julin N. Maloof and Cynthia Kenyon

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94143-0554, USA

Accepted 4 November 1997; published on WWW 17 December 1997

SUMMARY

The Ras signaling pathway specifies a variety of cell fates in many organisms. However, little is known about the genes that function downstream of the conserved signaling cassette, or what imparts the specificity necessary to cause Ras activation to trigger different responses in different tissues. In *C. elegans*, activation of the Ras pathway induces cells in the central body region to generate the vulva. Vulval induction takes place in the domain of the Hox gene *lin-39*. We have found that *lin-39* is absolutely required for Ras signaling to induce vulval development. During vulval induction, the Ras pathway, together with basal *lin-39* activity, up-regulates *lin-39* expression in vulval precursor cells. We find that if *lin-39* function is absent at this time, no vulval cell divisions occur. Furthermore, if *lin-39* is replaced with the posterior Hox gene *mab-5*, then posterior structures are induced instead of a vulva. Our findings suggest that in addition to permitting vulval cell divisions to occur, *lin-39* is also required to specify the outcome of Ras signaling by selectively activating vulva-specific genes.

Key words: Hox genes, Ras signaling, *C. elegans*, Vulval development, *lin-39*

INTRODUCTION

Two well-conserved strategies for determining patterns of cellular differentiation during development are the use of Ras-mediated intercellular signals to specify particular cell fates and the use of homeotic selector (Hox) genes to specify regional identity. Ras is a central member of the well-conserved receptor tyrosine kinase/Ras/MAP-Kinase signaling cassette (reviewed by Egan and Weinberg, 1993). In *C. elegans* and *Drosophila*, the Ras pathway regulates pattern formation in many tissues, including vulval cell fates in *C. elegans*, photoreceptor type in the *Drosophila* eye, and terminal fates in the *Drosophila* embryo (reviewed by Duffy and Perrimon, 1994; Eisenmann and Kim, 1994; Kayne and Sternberg, 1995; Wassarman et al., 1995). However, it is still not clear what gene products provide specificity to the Ras pathway; that is, what gene products determine the type of structure made in response to activation of the Ras pathway. Furthermore, few genes that act downstream of the conserved components of the pathway have been identified.

The Hox genes are best known for their role in specifying anterior/posterior (A/P) pattern in embryos (reviewed by McGinnis and Krumlauf, 1992; Botas, 1993; Krumlauf, 1994; Lawrence and Morata, 1994; Salser and Kenyon, 1994). These genes are expressed in broad stripes along the A/P axis, where they specify regional identity in all metazoans examined so far. Misexpression can cause homeotic transformations of one body region to another in *Drosophila* and *C. elegans*, and can cause homeotic skeletal defects in vertebrates. In both *Drosophila* and *C. elegans*, Hox genes are expressed dynamically, and this dynamic expression reflects a requirement for Hox genes at multiple times during development to specify diverse fates (Castelli and Akam, 1995; Salser and Kenyon, 1996). Both the *wingless* (Hoppler and Bienz, 1995) and *Sonic hedgehog* (Roberts et al., 1995) signaling molecules have been implicated in control of later Hox expression, but much remains to be learned about how dynamic expression is controlled.

Here we have investigated an interaction that takes place between Hox genes and the Ras pathway during cell fate specification in *C. elegans* vulval development. The Hox gene *lin-39*, the *C. elegans* homolog of *Drosophila sex-combs reduced (scr)*, specifies cell fates in the mid-body of the worm, where the vulva is located (Clark et al., 1993; Wang et al., 1993). The vulva arises from a set of six vulval precursor cells (VPCs), P3.p-P8.p, each of which has the potential to adopt a 1°, 2°, or 3° fate (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986). 1° and 2° cells divide multiple times to generate cells that will form the vulva; 3° cells are non-vulval: they divide once and join the epidermal syncytium. Vulval fates are specified both by a graded inductive signal from the gonadal anchor cell (AC) and by lateral signals between the VPCs (Fig. 1A) (Katz et al., 1995; Koga and Ohshima, 1995; Simske and Kim, 1995). The AC mediates vulval induction by producing a ligand that activates a conserved EGF-receptor/Ras/MAPK pathway in the VPCs (Fig. 1B) (Aroian et al., 1990; Beitel et al., 1990; Han and Sternberg, 1990; Hill and Sternberg, 1992; Eisenmann and Kim, 1994; Lackner et al., 1994; Wu and Han, 1994; Beitel et al., 1995; Kayne and Sternberg, 1995). A second signaling pathway involving *lin-12*, a *Notch* homolog, mediates lateral signaling between VPCs (Greenwald et al., 1983; Greenwald, 1985; Sternberg, 1988; Yochem et al., 1988).

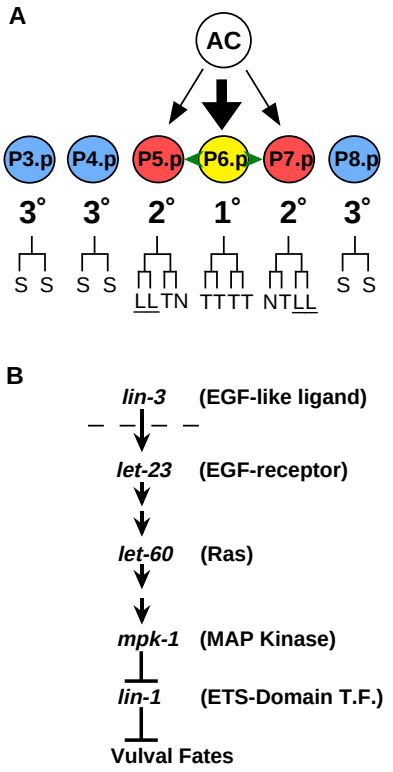

Fig. 1. Model for vulval induction. (A) Schematic of the AC and VPCs at the time of induction, and lineage diagram of the VPCs after induction. Anterior is to the left, ventral is down. Each VPC can adopt one of three fates, distinguishable by lineage analysis and cell morphology. The graded AC signal (black arrows), along with lateral signals between the VPCs (green arrowheads) ensure that the 3°-3°-2°-1°-2°-3° pattern of fates always occurs. The 1° and 2° fates are vulval fates and involve three rounds of division; the 3° fate is non-vulval and involves one round of division. Abbreviations: L, longitudinal division; underlining indicates progeny adherence to the cuticle at the L3 molt; T, transverse division; N, Pn.pxx nuclei that do not divide; S, Pn.p or Pn.px nuclei that do not divide and fuse with syncytial epidermis. (B) Vulval induction is mediated by a conserved EGF/Ras signaling pathway. *lin-3* encodes an EGF-like ligand which is expressed in the AC and signals the VPCs to begin vulval development (Hill and Sternberg, 1992); *let-23* encodes an EGF-receptor tyrosine kinase (Aroian et al., 1990); Ras, which acts as a switch to determine vulval fates is encoded by the *let-60* gene (Beitel et al., 1990; Han and Sternberg, 1990); *mpk-1/sur-1* encodes a MAP-kinase which functions to transmit the activating signal (Lackner et al., 1994; Wu and Han, 1994); and *lin-1* encodes an ETS-domain transcription factor which functions to inhibit vulval development (Beitel et al., 1995). Normally AC signals lead to the inactivation of *lin-1*, which then causes vulval development. Additional genes (not shown) include *lin-15*, part of a cell-non autonomous system that negatively regulates vulval induction (Clark et al., 1994; Huang et al., 1994), and *lin-12*, which mediates lateral signaling (Greenwald et al., 1983; Greenwald, 1985; Sternberg, 1988; Yochem et al., 1988).

Because the Hox gene *lin-39* is expressed in and functions to pattern the body region that generates the vulva, we suspected that it might be required for vulval development, and decided to investigate what its role might be. A recent study of the role of Hox genes in vulval development suggested that *lin-39* may be required for the VPCs to respond to Ras activation, and that the posterior Hox gene *mab-5* may antagonize vulval development (Clandinin et al., 1997). However, it has been difficult to assess the full role of *lin-39* in vulval development because *lin-39* is required for generation of the VPCs long before vulval induction takes place. In *lin-39* null mutants, the VPCs fuse with the surrounding epidermal syncytium and therefore cannot generate a vulva later in development.

We have used a *heat-shock-lin-39* construct to overcome the early fusion defect in *lin-39(–)* animals. This has enabled us to determine that *lin-39* plays a central role in vulval induction. At the time of vulval induction, the Ras pathway and preexisting LIN-39 protein together up-regulate *lin-39* gene expression. *lin-39* activity, in turn, is absolutely required in order for Ras signals to induce the vulva. If *lin-39* activity is not present at the time of Ras signaling, no divisions take place. In addition to permitting vulval cell divisions to occur, *lin-39* contributes specificity to the Ras pathway: expression of the incorrect Hox gene causes spatial homeotic transformations in the patterns that are induced in response to Ras activation. Together our findings suggest that in the VPCs, *lin-39* functions both in parallel to and downstream of the Ras pathway to selectively activate the expression of genes specific for vulval development.

MATERIALS AND METHODS

General methods and strains

Strains were maintained using standard methods (Brenner, 1974; Wood, 1988). Heat shock strains and *mab-5(e1751gf)* were maintained at 20°C; other strains were analyzed at 25°C. The following mutant alleles (Wood, 1988; or referenced below), were used:

LGI: *pry-1(mu38)* (J. N. M., unpublished). LGII: *let-23(sy97)* (Aroian and Sternberg, 1991). LGIII: *dig-1(n1321)* (Thomas et al., 1990), *mab-5(e1239)*, *mab-5(e2088)*, *mab-5(e1751gf)* (Hedgecock et al., 1987; Salser and Kenyon, 1992), *lin-39(mu26)* (Wang et al., 1993), *lin-39 (n709)* (Clark et al., 1993), *lin-39(n1760)* (Clark et al., 1993). LGIV: *lin-3(n378)*, *let-60(n1046gf)* (Ferguson and Horvitz, 1985; Beitel et al., 1990; Han et al., 1990), *unc-22(s7)*, *lin-1(e1026)*, *dpy-20(e1282)*. LGV: *him-5(e1467)*, *him-5(e1490)*. LGX: *lin-15(n309)*, *muIs9[hs-mab-5 unc-31(+)]* (Salser et al., 1993). Not yet assigned to a linkage group: *muIs23[hs-lin-39 dpy-20(+)]* (Hunter and Kenyon, 1995).

Antiserum preparation

PCR was used to delete the majority of the homeodomain (from N-175 to K-225) of a *lin-39* cDNA (1514A) (Wang et al., 1993); the resulting fragment was cloned into the $(His)_{10}$-containing pET16b (Novagen). The fusion protein was expressed and purified on Ni-NTA-agarose (Qiagen), and used to immunize two rabbits. Antibodies were affinity purified using LIN-39-$(His)_{10}$ protein immobilized on Ni-NTA-agarose; antibodies were eluted with Actisep (Sterogene). Purified antibodies show no reactivity against *lin-39(mu26)* or *lin-39(n1760)* larvae, and stain the nuclei of *hs-lin-39* worms brightly. Before use, affinity-purified antibodies were pre-adsorbed to *lin-39(n1760)* larvae as follows: *n1760* larvae were rinsed in dH_2O, placed in 100 μl aliquots in tubes in a 95°C PCR block for 10 seconds, and frozen in liquid N_2. Frozen worms were added to 15 ml tubes filled with methanol, incubated at room temperature for 1 minute, rinsed 3× in TBSTwE (137 mM NaCl, 2.7 mM KCl, 25 mM Tris at pH 7.7, 0.5% Tween 20, 5 mM EDTA), and placed in 2 volumes of block (1% BSA (Sigma #A9306), 5% swine serum (Cappel)). Worms were sonicated, and incubated at 37°C for 30 minutes. Purified

antibody was diluted 1:20 (final) and preadsorbed for 2 hours at 37°C. Debris was removed by spinning at 14,000 r.p.m. in an Eppendorf microfuge for 30 minutes, and the supernatant was used for immunostaining.

Immunostaining

For LIN-39 staining, larvae were rinsed 3 times with dH_2O. 20 μl of larvae were spotted onto poly-lysine-coated slides and covered with an 18 mm^2 coverslip. Slides were placed on a 95°C aluminum block (on a PCR machine) for 5 seconds, and then allowed to cool on the lab bench for <1 minute. Excess water was removed by aspiration, and then slides were frozen on an aluminum block on dry ice. Coverslips were pried off and the slides were placed in 25°C methanol for 3 minutes, rinsed 2× in TBSTwE, and then blocked for 30 minutes at 37°C. Larvae were incubated with primary antibody for 1-2 hours, washed 3× 20 minutes in TBSTwE, incubated with rhodamine-labelled donkey anti-rabbit IgG (1:50; Jackson Labs) for 1 hour, washed as above, taken through a TBSTwE/glycerol series, and mounted in 80% glycerol, 2% *n*-propyl gallate, 5 μg/ml DAPI.

For MH27 antibody labelled staining, fixation was as above, except that antibodies were diluted 1:100, and goat anti-mouse IgG (Cappel) was used as the secondary antibody.

Microscopy and laser ablation

Larvae were mounted (Wood, 1988) and observed using DIC (Nomarski) optics. Pn.p cells were observed beginning with the Pn.px or Pn.pxx stage. VPC fate assignment was done as described by Katz et al. (1995).

To observe autofluorescence of the hook, worms were viewed on a Zeiss Axiophot microscope fitted with a mercury lamp, using a standard fluorescein filter set.

Cell ablations were performed with a laser microbeam by standard methods (Bargmann and Avery, 1995). Z1 and Z4 ablations were performed within 4 hours of hatching. Control, unablated, animals were recovered from the same slides. Ablated animals were identified by the absence of germline nuclei as assayed by DAPI staining after fixation.

Heat shock of transgenic animals

A PTC-100 thermal cycler (MJ Research) was used to control heat pulses. 0- to 2-hour old larvae were placed on 35 mm NG plates that had been sanded to remove the ridges from the bottom of the plate for better thermal coupling. A 35 mm plate with a feedback temperature probe embedded in 4 ml of 2% agarose was used to increase reproducibility. Plates were placed directly on the 60-well thermal block (coated with mineral oil), and the machine was programmed as described below.

To assay vulval development in the absence of *lin-39* (Table 1B, and Fig. 3, *lin-39; lin-1* double mutants), we used the following program (times are given as hours:minutes): (1) 20°C 3:30; (2) 31°C 0:11; (3) 20°C 3:40; (4) back to step 2, 5 more times; (5) 20°C.

Variations of this program were used to provide *lin-39* later in development. A seventh, 11 minute, 31°C heat pulse was added either 3 hours and 40 minutes or 8 hours after the 6th pulse (Table 1C), or an 8 minute, 33°C heat pulse was added 2 or 3 hours after the 6th 31°C pulse (Table 1D).

To provide high levels of *lin-39* after Z1, Z4 ablation, animals were allowed to develop at 20°C for 21-23 hours (end of L2) or 23-25 hours (early L3) and then were given two 8 minute, 33°C heat pulses with 3 hours separating each pulse.

To provide *lin-39* to males (Figs 4B, 5D), we used the program: (1) 20°C 2:00 or 4:00; (2) 31°C 0:11; (3) 20°C 3:40; (4) back to step 2, 2 more times; (5) 32°C 0:10; (6) 20°C 2:40; (7) back to step 5, 2 more times; (8) 31°C 0:11; (9) 20°C 3:40; (10) back to step 8, 3 more times; (11) 20°C.

To provide *mab-5* to the central VPCs, and to reduce the amount of *lin-39(n709ts)* activity at the time of induction (Figs 4D, 5C), we used the program: (1) 15°C 38:00; (2) 20°C 2:00; (3) 31°C 0:11; (4) 25°C 2:45; (5) back to step 3, 1-3 more times. Step 2 was included because shifting from 15°C to 31°C directly was lethal.

RESULTS

Ras signaling increases *lin-39* expression in the VPCs

The Hox gene *lin-39* is known to be required early in development for generation of the VPCs (Clark et al., 1993; Wang et al., 1993). To determine whether *lin-39* might also be required later, at the time of vulval induction, we first used anti-LIN-39 antibodies to ask whether *lin-39* was expressed in the VPCs at this time. We found that before vulval induction occurs, *lin-39* was expressed uniformly in the VPCs. Laser ablation of the AC has shown that the AC signals the VPCs to initiate vulval development during early L3 (Kimble, 1981). We found that at the time of vulval induction, *lin-39* expression increased dramatically in P6.p, the VPC closest to the AC, which adopts the 1° vulval fate. P5.p and P7.p, which are further from the AC and which adopt 2° vulval fates, showed lower expression levels. The cells adopting the non-vulval 3° fate, P3.p, P4.p, and P8.p, showed the lowest levels of *lin-39* expression (Fig. 2A). Three experiments demonstrated that this expression pattern was governed by AC signals and the Ras pathway. First, the *dig-1(n1321)* mutation, which displaces the AC anteriorly, displaced the peak of *lin-39* expression coordinately (Fig. 2B). Second, ablation of the cells that generate the AC abolished the peak of expression (Fig. 2C). Third, Vulvaless (Vul) mutations that reduce Ras signaling decreased peak levels of Hox gene expression (Fig. 2D), whereas Multivulva (Muv) mutations that ectopically activate the signaling pathway caused strong expression in all VPCs (Fig. 2E).

How do Ras signals control LIN-39 levels? We have observed that *hs*-LIN-39 protein is expressed and decays uniformly in the VPCs (data not shown), suggesting that Ras signals regulate *lin-39* transcription or translation, rather than degradation. Together, these findings showed that *lin-39* expression is up-regulated by the Ras signaling pathway, and suggested that *lin-39* might be required at the time of vulval induction.

lin-39 and Ras signaling are both required for vulval induction

In *lin-39(–)* mutants, cells that would normally become VPCs instead fuse with the surrounding epidermal syncytium well before the time of vulval induction (Clark et al., 1993; Wang et al., 1993). Therefore, to determine whether *lin-39* activity was also required later, at the time of vulval induction, we used a *heat-shock-lin-39* chimeric construct *(hs-lin-39)* to control *lin-39* expression levels in *lin-39(–)* animals. We gave repeated heat pulses early in development, but stopped the heat pulses to allow LIN-39 levels to drop by the time of vulval induction. Under these conditions, many VPCs remained unfused; however, we found that they adopted non-vulval fates (Table 1B, especially note P6.p descendants). Later and stronger heat pulses restored wild-type vulval development (Table 1C,D). Thus, *lin-39* is not only required early to prevent fusion of the VPCs, it is also required later to allow VPCs to generate vulval cell lineages.

Table 1. Response of *lin-39(–)* animals to pulses of *hs-lin-39*

Animal no.	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
A. Wild type						
	SS	SS	LLTN	[TTTT]	NTLL	SS
B. *hs-lin-39* (low)						
1.	S	S	S	SS	S	S
2.	S	S	S	SS	S	S
3.	S	S	S	SS	S	S
4.	S	S	SS	SS	S	S
5.	S	S	SS	SS	S	S
6.	S	SS	SS	SS	SSS	S
7.	S	SS	SS	SS	SSS	SS
8.	SS	SS	SS	SS	TT S	SS
9.	S	S	S	SS	TL S	S
10.	SS	S	ULLN	SS	UUUU	SS
11.	S	S	S	SS	LLUU	S
12.	S	S	S	SS	TLUU	S
13.	S	S	S	SS	TUUU	S
14.	S	S	S	SS	OONU	S
15.	S	S	S	UU S	LLUU	S
16.	SS	SS	SS	S UL	SS	SS
17.	SS	SS	S TN	S LT	NTUU	SS
18.	S	S	S	NTLN	UU S	S
19.	S	S	S	[TUUT]	S	S
20.	S	S	SS	[TUTN]	NLUU	SS
21.	S	SS	S NT	[TTTT]	NT S	SS
C. *hs-lin-39* (medium)						
	P3.p	**P4.p**	**P5.p**	**P6.p**	**P7.p**	**P8.p**
22.	SS	S	UULL	SS	SS	S
23.	S	S	SS	SS	LLUU	S
24.	S	S	UUUU	SS	UUUU	S
25.	S	SS	SS	S LT	NTUU	S
26.	SS	SS	S TL	S TT	NTLL	SS
27.	S	S	SS	TLUU	S	S
28.	S	S	SS	LLUU	SS	S
29.	S	S	S	[LTTL]	S	S
30.	SS	SS	SS	[TLNT]	SS	SS
31.	S	S	S	[TTTT]	S	S
32.	S	SS	UUTN	[TTTL]	S	S
33.	S	SS	SS	[NTTT]	NUUU	S
34.	S	S	S	[TTTL]	NTLU	S
35.	S	S	S	[TTTT]	TLTU	S
36.	SS	SS	SS	[TTTT]	NTLU	SS
37.	D	D	S UU	[TTTT]	LLUU	D
38.	S	S	S TN	[TTTT]	NTUU	SS
39.	S	S	S UN	[TTOT]	NTLU	S
40.	S	SS	S NT	[TTTT]	NTUU	SS
41.	S	S	UULN	[TTTT]	NTUL	S
42.	S	SS	ULLN	[TTTT]	NLUU	SS
D. *hs-lin-39* (high)						
43.	S	S	S	SS	LLLL	S
44.	SS	SS	SS	SS	LTLL	S
45.	S	SS	S	TU S	UUOL	SS
46.	SS	SS	SS	TTNL	UU S	SS
47.	S	S	S	[TTTT]	TLUU	S
48.	SS	S	S	[TTTT]	NTLL	S
49.	SS	S	S	[LTTT]	NTLL	SS
50.	S	S	SS	[TTTT]	NTUU	S
51.	S	SS	UUTN	[TTTT]	NTLU	SS
52.	SS	SS	UUTN	[TTTT]	NTLO	SS
53.	S	SS	ULTN	[TTTT]	NTUU	SS
54.	SS	SS	ULTN	[TTTT]	NTLS	SSS
55.	SS	SS	LOTN	[TTTT]	NTLL	SS
56.	SS	SS	LLTN	[TTTT]	NTLL	S
57.	SS	SS	LLTN	[TTTT]	NTLL	SS

Hermaphrodite Pn.p lineages are shown for the following genotypes:

(A) Wild type.

(B) *lin-39(–); hs-lin-39* animals given six 31°C pulses.

(C) *lin-39(–); hs-lin-39* given seven 31°C pulses.

(D) *lin-39(–); hs-lin-39* given six 31°C pulses followed by a 33°C pulse (see Materials and Methods for details).

See Fig. 1 legend for abbreviations. Additional abbreviations: U, a Pn.pxx nuclei that did not divide but did not take on the characteristic morphology of an N cell; O, divided along an oblique axis; D, did not observe. Box indicates a 1° fate. On average, a greater amount of time elapsed between the final pulse of *hs–lin–39* expression and the onset of Pn.p divisions in animals with less vulval development, suggesting that the animals with less vulval development had less LIN–39 at the time of Pn.p division (also supported by antibody staining, data not shown). We used the monoclonal antibody MH27, which recognizes an antigen at apical cell boundaries, to determine if our heat shock regime prevented VPC fusion. We stained a fraction of the animals given the "low" heat shock regime and found that P(5-7).p were unfused in all of the animals (*n*=32).

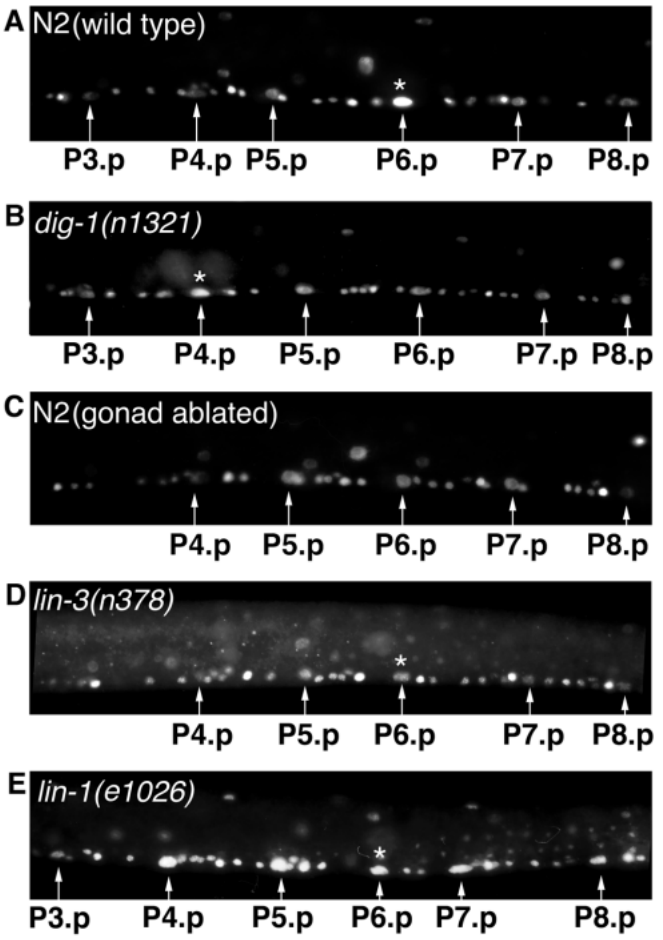

Fig. 2. *lin-39* is expressed in an AC and Ras dependent gradient in the VPCs. (A) Wild-type larva, showing strong LIN-39 antibody staining in P6.p and weaker staining in the VPCs to either side. Staining in the neurons of the ventral cord can also be seen (small nuclei between the VPCs). (B) *dig-1(n1321)* larva. In *dig-1* mutants, the gonad often shifts to the anterior, thereby causing anterior vulval induction. In this animal the gonad was centered over P4.p, the cell expressing *lin-39* most strongly. (C) Wild-type larva in which the somatic gonad precursor cells, Z1 and Z4, have been ablated; these animals have no AC and are therefore not able to induce a vulva. In animals with no AC, the LIN-39 antibody staining is reduced in P6.p. (D) *lin-3(n378)* mutant showing weaker expression in the VPCs, especially P6.p, as compared to wild type. The AC induces the vulva via an EGF-like ligand encoded by the *lin-3* gene. Similar staining was seen in *let-23(sy97)* mutant animals, which have a mutation in the EGF-receptor gene (data not shown). (E) *lin-1(e1026)* mutant larva showing increased expression in the VPCs. *lin-1* encodes an ETS-like transcription factor which inhibits vulval induction. Similar staining was seen in two other strains with activated Ras pathways: *lin-15(n309)* and *let-60-ras(n1046gf)*(data not shown). About 50% of AC-ablated and signaling-defective larvae show a weak gradient of LIN-39 across the VPCs (the very high levels of LIN-39 seen in wild-type animals are never seen in these mutants). This suggests that there may be a Ras-independent mechanism for biasing *lin-39* expression in the VPCs. *****Indicates approximate AC position.

Where in the Ras signaling pathway does *lin-39* act? In *C. elegans*, as in other organisms, the Ras signaling cascade leads to modification of an ETS domain transcription factor (Beitel et al., 1995). The ETS homolog that functions during vulval development is the gene *lin-1*, which acts at the downstream-most position of the conserved signaling cassette to inhibit vulval development. Activation of the Ras pathway by AC signals leads to inactivation of *lin-1*, which, in turn, promotes vulval development. We found that *lin-39* expression was increased in *lin-1(–)* mutants (Fig. 2E). This finding suggested that control of *lin-39* expression by the Ras pathway was mediated, directly or indirectly, by *lin-1*, and identified *lin-39* as a downstream target of the Ras signaling cassette.

Animals lacking *lin-1* activity have a Multivulva phenotype: in *lin-1(–)* mutants, all the VPCs generate vulval cell lineages in an anchor-cell independent fashion. Because *lin-39* expression increases in *lin-1(–)* mutants, it seemed likely that LIN-39 protein would act downstream of *lin-1*, and thus be required for the Multivulva phenotype of *lin-1*. To test this, we used early pulses of *hs-lin-39* in *lin-39(–); lin-1(–)* mutants, as described above, to prevent VPC fusion, and then stopped the heat pulses to allow LIN-39 levels to fade. No vulval development took place (68/70 cells adopted the non-vulval, 3° fate). This indicated that *lin-39* activity is required for the *lin-1* Multivulva phenotype, and thus for aspects of vulval development that take place after the Ras pathway inactivates *lin-1*.

We next investigated whether high levels of *lin-39* expression might be sufficient to induce vulval development in the absence of Ras signaling. To test this, we inactivated the Ras pathway by killing the anchor cell, and then administered high, uniform levels of *LIN-39* by using *hs-lin-39*. We found that no vulval cell divisions occurred (n=37). In addition, when high uniform levels of *LIN-39* were administered in animals with intact anchor cells, no ectopic vulval induction was seen (Table 1D). These findings indicated that *lin-39* alone cannot trigger vulval development. Thus, the Ras pathway must have other functions in vulval development in addition to inducing *lin-39* expression. These findings also ruled out a possible role for *lin-39* that had been suggested by the graded *lin-39* expression pattern; namely, that, different levels of *lin-39* are used to specify alternative 1°, 2°, or 3° vulval fates.

lin-39 specifies its own up-regulation in response to Ras signaling

The finding that *lin-39* expression was up-regulated by the Ras pathway was surprising, and it also raised an apparent paradox: Ras is required in many cells during *C. elegans* development (Han et al., 1990; Han and Sternberg, 1990; Chamberlin and Sternberg, 1994), but Ras signaling up-regulates *lin-39* only in the VPCs, not in other cells. What specifies that *lin-39* is controlled by Ras signaling in the VPCs? *lin-39* itself is expressed at low levels in the VPCs before Ras activation (Fig. 3A); thus, one possibility was that basal *lin-39* activity may be required for up-regulation of *lin-39* in response to the activation of Ras. We tested this hypothesis using *n2110*, a *lin-39* allele that greatly reduces gene activity but still expresses wild-type levels of LIN-39 protein in many cells as detected by immunofluorescence. We expressed *hs-lin-39* early to prevent fusion of the VPCs and then examined the pattern of endogenous *lin-39(n2110)* expression at the time of AC signaling. We found that in *n2110*, *lin-39* expression was nearly uniform in the central VPCs, P5.p, P6.p, and P7.p (Fig. 3B; compare to *hs-lin-39* animals with *lin-39(+)* in Fig. 3C). The *n2110* mutation is a single base change in the

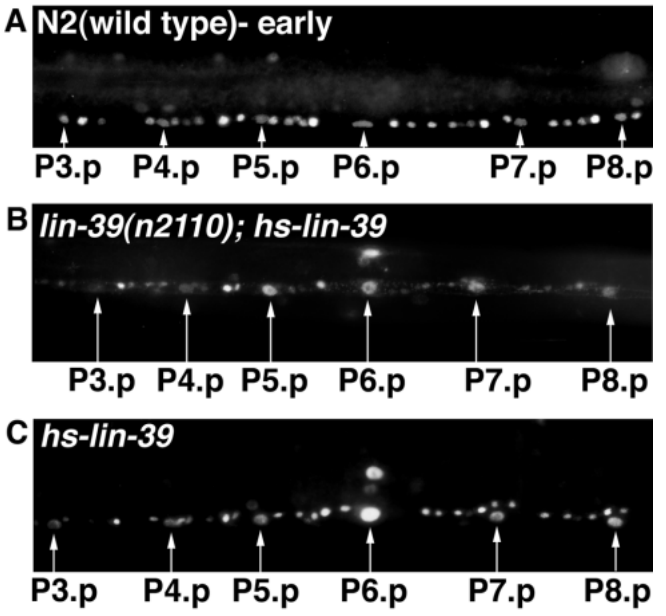

Fig. 3. *lin-39* is required for its own up-regulation in response to Ras signaling. (A) In wild-type larvae, *lin-39* is expressed uniformly at 'basal' levels in the VPCs before vulval induction. (B) *lin-39(n2110); hs-lin-39* mutant showing relatively uniform levels of expression in P(5-7).p, similar to the AC-ablated animal (Fig. 2C). Pulses of *hs-lin-39* were used early during development to keep the VPCs unfused. At the time of this photo, LIN-39 is only detectable in the central body region, showing that the ubiquitously expressed *hs*-LIN-39 has been degraded (additional experiments have shown that *hs*-LIN-39 is degraded uniformly along the body axis; data not shown). Note that mutant *(n2110)* LIN-39 protein is present at high levels in other cells, suggesting that the low level of expression in the VPCs is not due to an inherent instability of the mutant protein. (C) Control *lin-39(+); hs-lin-39* animal pulsed and stained in parallel to the animal in Fig. 3B. In the presence of *lin-39(+)*, the normal gradient of *lin-39* expression is seen.

homeodomain coding region (Clark et al., 1993) and thus probably acts by reducing the activity of LIN-39 protein. Therefore early basal levels of *lin-39* activity appear to be required for up-regulation of *lin-39* expression in response to Ras signaling.

lin-39 contributes specificity to the Ras signaling pathway

How Ras signaling initiates different developmental programs in different tissues is not well understood. Hox genes function as homeotic selector genes during development, distinguishing between alternative cell fates in a position-specific manner. We therefore hypothesized that, in addition to allowing further development to take place, *lin-39* might also influence the type of structure generated by the VPCs in response to activation of the Ras pathway. The male equivalence group, consisting of P(9-11).p, the posterior homologs of the hermaphrodite VPCs, gives rise to the hook, a structure used by the male when mating, and the pre-anal ganglion, a group of neurons important for mating (referred to here collectively as the pre-anal group or PAG). There are many similarities between the development of the PAG and the vulva: both are generated from Pn.p cells that undergo three rounds of division at the end of L3, and both are affected by the *lin-12* lateral signaling pathway (Greenwald et al., 1983). In addition, both structures are affected by mutations that perturb Ras signaling, although the role of Ras in PAG development is more limited than in vulval development (Paul Sternberg, personal communication; data not shown).

The vulval precursors express the Hox gene *lin-39*, whereas the PAG precursors express *mab-5*, the Hox gene that functions in the body region posterior to the *lin-39* domain (Salser et al., 1993). Because these structures develop from similar cells and are patterned by some of the same signaling systems, we reasoned that Hox genes might be required to specify alternative organ types. We tested this hypothesis in two ways: first by mis-expressing *lin-39* in the posterior and second by mis-expressing *mab-5* in the central body. We expressed *hs-lin-39* in *mab-5(–)* males to determine if *lin-39* could cause posterior male cells to adopt vulval instead of PAG fates. We used three vulva-specific features to assay the effects of *lin-39* mis-expression. First, morphology: vulval cells invaginate in a unique way (compare Fig. 4A and C); second, plane of cellular division: some vulval cells, known as T cells, divide along the transverse (L/R) axis, whereas PAG cells do not (Fig. 5A,B); and third, detachment from the cuticle: a subset of both vulval and PAG cells detach from the cuticle in characteristic patterns (attached cells are underlined in Fig. 5A,B,D; see legend). We found that ectopic expression of *hs-lin-39* in the PAG-precursor cells caused their descendants to form vulva-like invaginations (Fig. 4B), induced some T divisions characteristic of vulval cells, and caused detachment patterns reminiscent of those seen in the vulval secondary lineage (Fig. 5D). Although the transformation was not complete, we conclude that replacing *mab-5* with *hs-lin-39* can cause posterior male equivalence group cells to adopt vulval characteristics.

We next asked whether the reciprocal situation, expressing *mab-5* in the normal *lin-39* domain, might cause vulval cells to adopt PAG fates. One of the male equivalence group descendants generates the hook, an arrowhead shaped structure used by males in mating. The hook is autofluorescent when illuminated with light of the appropriate wavelength (Link et al., 1988; see Materials and Methods) (Fig. 4F) whereas the vulva is not (Fig. 4E); thus, autofluorescence can be used as a marker for the hook. We looked for autofluorescence in the vulval region of hermaphrodites in two strains that ectopically express *mab-5* in the mid-body (Salser and Kenyon, 1992; J. N. M. and C. K. in preparation). About 4% of *mab-5(e1751gf)* and 24% of *pry-1(mu38)* mutant hermaphrodites show autofluorescence, in some cases hook-shaped, near their vulva (Fig. 4G; data not shown). The autofluorescence is likely due to *mab-5*, since no autofluorescence is seen in a *pry-1(mu38); mab-5(e2088lf)* double mutant (J. N. M. and C. K., in preparation). Furthermore, no hook-like autofluorescence was seen in *mab-5(e1751gf)* hermaphrodites after ablation of the AC precursors, which shows that AC signals are needed for ectopic hooks (n=165, P<0.003), and suggests that *mab-5* specifies ectopic hook development by changing the fates of cells generated in response to the Ras pathway. We did not see any changes in the pattern of vulval divisions in these animals. However, these experiments were performed in the presence of wild-type *lin-39*, which we thought might be interfering with the ectopic *mab-5*.

To ask whether ectopic *mab-5* expression might cause a

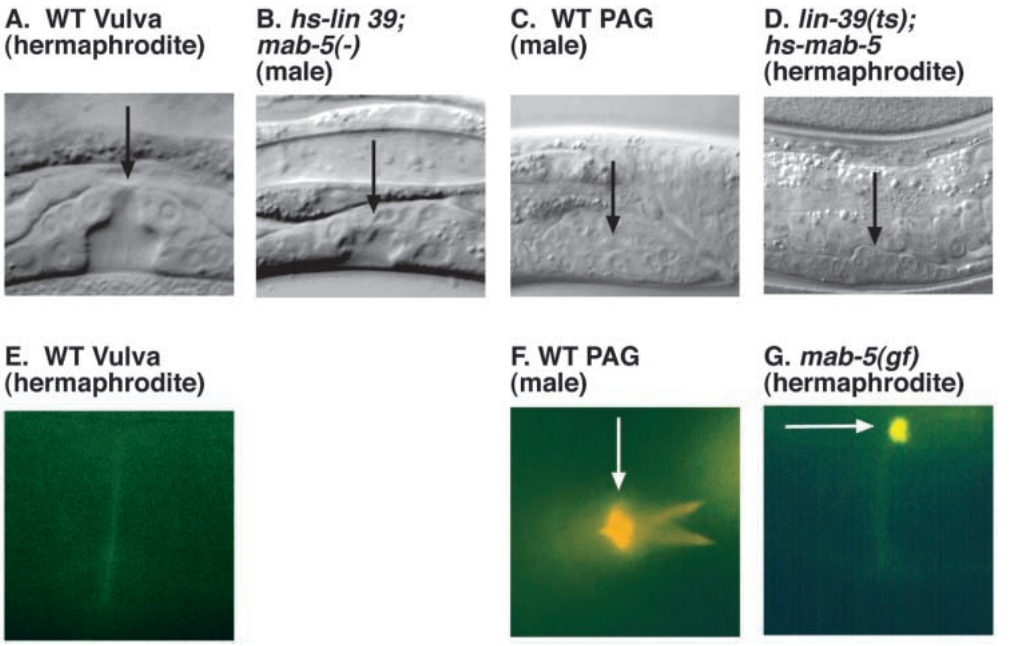

Fig. 4. Hox genes play a role in specifying organ identity. (A) A wild-type hermaphrodite showing vulval invagination (arrow) during L4. (B) Representative *mab-5(–)* male after *hs-lin-39.* Descendants of P10.p and P11.p formed an invagination (arrow) reminiscent of the invagination seen in the wild-type hermaphrodite vulva. (C) A wild-type male PAG during L4. The P11.p descendants have detached from the cuticle but do not invaginate (n=13). The arrow points to the middle of the area containing the P10.p and P11.p descendants. (D) An early L4 *lin-39(ts); hs-mab-5* hermaphrodite showing that the transformed descendants of P5.p-P7.p (arrow) do not form a vulval invagination. (E) Epifluorescence image of adult wild-type vulva. There are no bright spots of autofluorescent near the vulva. (F) Epifluorescence image of adult male tail. The arrow points to the autofluorescent hook. (G) Epifluorescence images of a *mab-5(e1751gf)* hermaphrodite showing an ectopic hook (arrow) in the vulval region; *pry-1(mu38)* animals look similar (not shown). 6/146 *e1751* and 7/22 *mu38* hermaphrodites showed autofluorescence, whereas 0/165 AC precursor-ablated *e1751* hermaphrodites and 0/25 *mu38; e2088* hermaphrodites showed autofluorescence.

more complete transformation in the absence of *lin-39,* we decided to remove *lin-39* activity and then examine the effects of ectopic *mab-5* expression on vulval development. To do this, we used *lin-39(n709),* a temperature-sensitive allele, along with a *hs-mab-5* construct. Young worms were grown at the permissive temperature (15°C) to allow *lin-39* activity to keep the VPCs unfused. Shortly before vulval induction, the temperature was raised to 25°C to reduce *lin-39* activity, and then 31°C heat pulses were given to induce *hs-mab-5* expression. This regime resulted in a dramatic transformation of vulval to PAG fates as assayed by several criteria. Unlike wild-type vulval cells, the transformed cells did not invaginate (Fig. 4D). In contrast to wild-type or control *(n709)* vulval lineages, no T divisions occurred; instead, *hs-mab-5* promoted a pattern of L divisions similar to that seen in PAG lineages (Fig. 5C). In three of four *hs-mab-5* animals, P6.p underwent a division pattern characteristic of P11.p in the PAG: one daughter divided obliquely (* in Fig 5), and the other daughter generated a descendant that did not divide again (N in Fig. 5). Two animals produced a P5.p granddaughter that underwent an additional division, resulting in a P5.p lineage identical to that of a normal P10.p in the PAG. These division patterns were never seen in wild-type or control vulval lineages. Thus replacing *lin-39* with *mab-5* causes a transformation of vulval cell fates to PAG cell fates. Together with the *hs-lin-39* data, these results suggest that *lin-39* is required in the vulva to specify that vulval fates are adopted as an outcome of Ras signaling.

DISCUSSION

In this study, we have examined the role of the Hox gene *lin-39* in the development of the *C. elegans* vulva. We have found that this Hox gene is a downstream target of the Ras pathway, that it is required for all vulval cell divisions, and that it imparts specificity to the signaling system.

Ras signaling can up-regulate *lin-39* expression

Mechanisms for controlling Hox expression in fields of cells late in development are now beginning to be identified. For example, in the chick, expression of Hox genes in the hindgut is under control of *Sonic hedgehog* (Roberts et al., 1995) and, in *Drosophila,* Wnt signaling controls the expression of the Hox genes *Ultrabithorax* in the visceral mesoderm and *labial* in the midgut endoderm (Thuringer and Bienz, 1993; Hoppler and Bienz, 1995). Our findings identify a different signaling pathway, Ras, as being able to control Hox expression late in development. Given that the Ras pathway controls *lin-39* expression during vulval development, and the prevalence of the EGF/Ras pathway in cell fate specification, it seems possible that Hox genes will be found under EGF/Ras control in other organisms as well.

lin-39 is required for vulval development

Little is known about how activation of the conserved Ras signaling cassette leads to specific development of the *C. elegans* vulva; this work shows that the Hox gene *lin-39* plays an important role in this process. We have shown that *lin-39* is absolutely required in order for Ras signaling to induce a vulva: without *lin-39* activity, no VPC cell division or vulval development takes place. Our genetic analysis suggests that, at a functional level, *lin-39* acts in parallel to the Ras pathway during vulval development. *lin-39* activity is still required for vulval development even if *lin-1,* the transcriptional regulator that is inactivated by Ras signaling, has been eliminated by

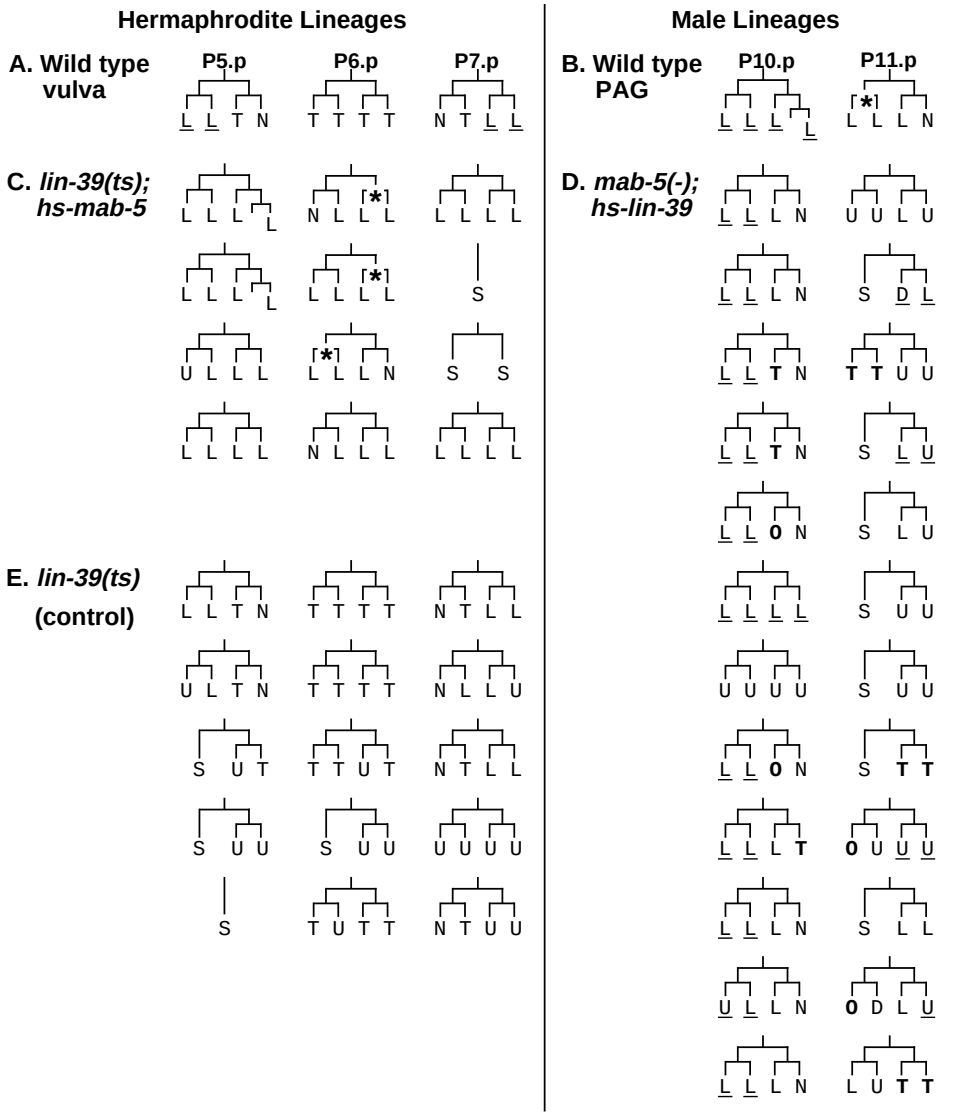

Fig. 5. Hox gene mis-expression can cause vulval and PAG lineage transformations. (A) Wild-type vulval lineage (see abbreviations in Fig. 1 and Table 1). In vulval 2° lineages, the outer cells, the L cells (and their descendants) stay attached to the cuticle (denoted by the underline), whereas the N and T cells detach from the cuticle. (B) Wild-type PAG lineage. Note that there are no T divisions and that all descendants of P10.p stay attached, and all descendants of P11.p detach. In addition, P11.pa divides obliquely (denoted by *) (n=13). (C) Lineages of P(5-7).p in *lin-39(n709); hs-mab-5* hermaphrodites. *hs-mab-5* transforms the vulval lineages to PAG-like lineages. Adherence to the cuticle was not scored in these animals (so no cells are underlined), but the cells failed to undergo the normal vulval invagination. It is interesting to note that the 1° PAG fate (adopted by P11.p in wild type) was adopted by P6.p in at least three of the four *hs-mab-5* animals. Since P6.p normally adopts the 1° vulval fate due to its proximity to AC signals, these results suggest that Ras signals pattern the ectopic PAG fates in a manner similar to wild-type, and furthermore that localized activation of the Ras pathway may be important for determining the fate of P11.p in wild-type males. See Materials and Methods for heat-shock regime. (D) Lineages of P10.p and P11.p in *mab-5(–)* males after *hs-lin-39*. Vulva-like T or O divisions (denoted by bold type) occurred in 5/12 males lineaged. Furthermore, in 10/12 *hs-lin-39* males, P10.p gave rise to two L cells that remained attached and an LN, TN, or ON combination that detached, thereby producing a vulva-like 2° lineage and detachment pattern. Only animals with multiple Pn.p divisions are shown. (E) Lineages of P(5-7).p in control *lin-39(n709)* animals without the *hs-mab-5* construct. These animals were given the same heat-shock regime as those in C. The lineages are still characteristically vulval, showing that the PAG-like lineages in (C) are due to *hs-mab-5*, rather than reduced *lin-39* activity. Adherence to the cuticle was not scored in these animals, although the central cells did invaginate (not shown).

mutation. Conversely, the Ras pathway is still required for vulval development even if high, uniform levels of *lin-39* are administered artificially. In addition, since *lin-39* expression is also up-regulated by the Ras pathway, *lin-39* can also be considered to be a downstream target of the Ras pathway.

lin-39 imparts specificity to Ras signal transduction during vulval development

lin-39 can promote vulva-like lineages when expressed ectopically in the male tail, and the posterior Hox gene *mab-5* can transform the vulval cells, causing them to make posterior structures instead. These findings indicate that *lin-39* instructs the VPCs to adopt vulval fates, rather than an alternative fate, in response to Ras signals. It is important to emphasize that the homeotic transformations that we observed when *lin-39* was expressed in the posterior body region were not complete. For this reason, we believe that additional specificity factors are likely to be required as well.

How might *lin-39* confer specificity to the Ras pathway? As described above, it was known previously that AC signals lead to the inactivation of *lin-1*, and that *lin-1* inhibits vulval development, probably by acting as a transcriptional repressor. Our findings indicate that *lin-1* represses *lin-39*. In addition, *lin-1* must repress other genes, since ectopic *lin-39* expression cannot overcome the requirement for AC signals. One attractive model to explain these findings is the following: in the absence of AC signals, *lin-1* represses expression of three types of genes: (i) *lin-39*, (ii) genes directly involved in vulval development, and (iii) genes with unrelated functions (for example, those involved in PAG fates) (Fig. 6A). When *lin-1* is inactivated by AC signaling, all three types of genes become competent for expression. Some LIN-39 protein is already present even in the

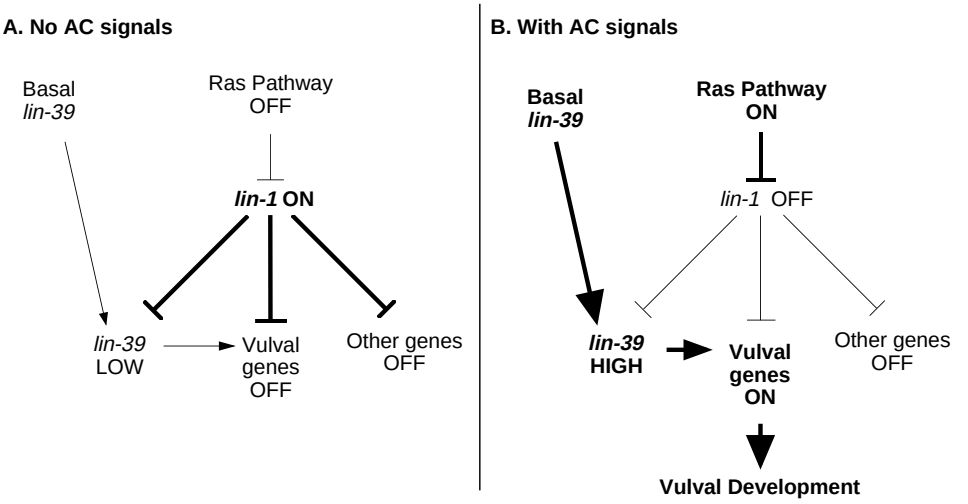

Fig. 6. Model for vulval induction. (A) In the absence of AC signals, *lin-1* is active and represses a number of genes including *lin-39* and vulval genes. (B) AC signals inactivate *lin-1*, resulting in an increase in *lin-39* expression and enabling the activation of vulval genes by *lin-39*. Basal *lin-39* is required for up-regulation of *lin-39* expression in response to Ras signaling.

absence of Ras signaling, and this basal LIN-39 is able to activate strong expression of the *lin-39* gene in the absence of *lin-1*. In addition, *LIN-39* is now able selectively to activate those genes that are vulva-specific, thereby initiating vulval development (Fig. 6B). In this way, AC signaling limits gene activation to the central VPCs, and *lin-39* activity ensures that only vulva-specific genes are activated. This model is supported by the finding that *lin-39* is required for its own up-regulation in response to Ras signaling. Specifically, this result identifies *lin-39* itself as an example of a vulva-specific gene whose expression requires both Ras activation and *lin-39* activity, and thus clearly demonstrates that *lin-39* can act to select vulva-specific genes for activation by the Ras pathway. We imagine that when we replaced *lin-39* with *mab-5*, a different set of genes was activated in response to *lin-1* inactivation.

In summary, we have found that the Hox gene *lin-39* plays two roles in vulval development. First, this Hox gene is absolutely necessary for vulval cell divisions to occur following activation of the Ras pathway. Interestingly, blocking expression of specific Hox genes or Ras can inhibit division of human melanoma cells (Care et al., 1996; Ohta et al., 1996), suggesting that Hox genes may permit Ras-mediated cell division in higher organisms as well. Second, *lin-39* function contributes specificity to the Ras signaling pathway, probably by selecting vulva-specific genes for transcriptional activation. Homeotic selector genes, rather than novel factors, may impart specificity to signaling pathways in other cases as well.

We thank Raffi Aroian, Cori Bargmann, Stacey Harmer, Lee Honigberg, Craig Hunter, Gregg Jongeward, Steve Salser, Mary Sym, and Ilan Zipkin, for useful discussion and comments on the manuscript. Helen Chamberlain and Paul Sternberg kindly provided us with *sy97*, *n1046*, and *n378*. Our reviewers provided motivation to do some very interesting experiments. Some strains were obtained from the Caenorhabditis Genetics Center, which is supported by the National Center for Research Resources of the National Institutes of Health. J. N. M. was supported in part by a HHMI pre-doctoral fellowship. This work was supported by NIH grant RO1 GM37053 to C. K.

REFERENCES

Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily [see comments]. *Nature* **348,** 693-699.

Aroian, R. V. and Sternberg, P. W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. *Genetics* **128,** 251-267.

Bargmann, C. I. and Avery, L. (1995). Laser killing of cells in *Caenorhabditis elegans*. *Methods Cell Biol* **48,** 225-250.

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction [see comments]. *Nature* **348,** 503-509.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev*. **9,** 3149-62.

Botas, J. (1993). Control of morphogenesis and differentiation by HOM/Hox genes. *Curr. Opin. Cell. Biol.* **5,** 1015-1022.

Brenner, S. (1974). The genetics of *C elegans*. *Genetics* **77,** 71-94.

Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C. and Colombo, M. P. (1996). HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol. Cell Biol.* **16,** 4842-4851.

Castelli, G. J. and Akam, M. (1995). How the Hox gene *Ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development* **121,** 2973-2982.

Chamberlin, H. M. and Sternberg, P. W. (1994). The *lin-3/let-23* pathway mediates inductive signalling during male spicule development in *Caenorhabditis elegans*. *Development* **120,** 2713-2721.

Clandinin, T. R., Katz, W. S. and Sternberg, P. W. (1997). *Caenorhabditis elegans* HOM-C genes regulate the response of vulval precursor cells to inductive signal. *Dev. Biol.* **182,** 150-161.

Clark, S. G., Chisholm, A. D. and Horvitz, H. R. (1993). Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. *Cell* **74,** 43-55.

Clark, S. G., Lu, X. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137,** 987-997.

Duffy, J. B. and Perrimon, N. (1994). The torso pathway in *Drosophila*: lessons on receptor tyrosine kinase signaling and pattern formation. *Dev. Biol*. **166,** 380-395.

Egan, S. E. and Weinberg, R. A. (1993). The pathway to signal achievement [news]. *Nature* **365,** 781-783.

Eisenmann, D. M. and Kim, S. K. (1994). Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opin. Genet. Dev*. **4,** 508-516.

Ferguson, E. L. and Horvitz, H. R. (1985). Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics* **110,** 17-72.

Greenwald, I. (1985). *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. *Cell* **43,** 583-590.

Greenwald, I. S., Sternberg, P. W. and Horvitz, H. R. (1983). The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. *Cell* **34,** 435-444.

Han, M., Aroian, R. V. and Sternberg, P. W. (1990). The *let-60* locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*. *Genetics* **126,** 899-913.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63,** 921-931.

Hedgecock, E. M., Culotti, J. G., Hall, D. H. and Stern, B. D. (1987). Genetics of cell and axon migrations in *Caenorhabditis elegans*. *Development* **100,** 365-382.

Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans* [see comments]. *Nature* **358,** 470-476.

Hoppler, S. and Bienz, M. (1995). Two different thresholds of wingless signalling with distinct developmental consequences in the *Drosophila* midgut. *EMBO J.* **14,** 5016-5026.

Huang, L. S., Tzou, P. and Sternberg, P. W. (1994). The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. *Mol. Biol. Cell* **5,** 395-411.

Hunter, C. P. and Kenyon, C. (1995). Specification of anteroposterior cell fates in *Caenorhabditis elegans* by *Drosophila* Hox proteins. *Nature* **377,** 229-232.

Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precursor fates [see comments]. *Cell* **82,** 297-307.

Kayne, P. S. and Sternberg, P. W. (1995). Ras pathways in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* **5,** 38-43.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol*. **87,** 286-300.

Koga, M. and Ohshima, Y. (1995). Mosaic analysis of the *let-23* gene function in vulval induction of *Caenorhabditis elegans*. *Development* **121,** 2655-2666.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78,** 191-201.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homolog, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev*. **8,** 160-173.

Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in *Drosophila* segmentation and pattern formation. *Cell* **78,** 181-189.

Link, C. D., Ehrenfels, C. W. and Wood, W. B. (1988). Mutant expression of male copulatory bursa surface markers in *Caenorhabditis elegans*. *Development* **103,** 485-495.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68,** 283-302.

Ohta, Y., Kijima, H., Ohkawa, T., Kashani, S. M. and Scanlon, K. J. (1996). Tissue-specific expression of an anti-ras ribozyme inhibits proliferation of human malignant melanoma cells. *Nucl.c Acids Res.* **24,** 938-942.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C. (1995). *Sonic hedgehog* is an endodermal signal inducing *Bmp-4* and Hox genes during induction and regionalization of the chick hindgut. *Development* **121,** 3163-3174.

Salser, S. J. and Kenyon, C. (1992). Activation of a *C. elegans Antennapedia* homologue in migrating cells controls their direction of migration. *Nature* **355,** 255-258.

Salser, S. J. and Kenyon, C. (1994). Patterning *C. elegans*: homeotic cluster genes, cell fates and cell migrations. *Trends Genet.* **10,** 159-164.

Salser, S. J. and Kenyon, C. (1996). A *C. elegans* Hox gene switches on, off, on and off again to regulate proliferation, differentiation and morphogenesis. *Development* **122,** 1651-1661.

Salser, S. J., Loer, C. M. and Kenyon, C. (1993). Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system. *Genes Dev*. **7,** 1714-1724.

Simske, J. S. and Kim, S. K. (1995). Sequential signalling during *Caenorhabditis elegans* vulval induction. *Nature* **375,** 142-146.

Sternberg, P. W. (1988). Lateral inhibition during vulval induction in *Caenorhabditis elegans*. *Nature* **335,** 551-554.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans*. *Cell* **44,** 761-772.

Sulston, J. and Horvitz, H. (1977). Post-embryonic cell lineages of the nematode, *C elegans*. *Dev. Biol.* **56,** 110-156.

Thomas, J. H., Stern, M. J. and Horvitz, H. R. (1990). Cell interactions coordinate the development of the *C. elegans* egg-laying system. *Cell* **62,** 1041-1052.

Thuringer, F. and Bienz, M. (1993). Indirect autoregulation of a homeotic *Drosophila* gene mediated by extracellular signaling. *Proc. Natl. Acad. Sci. USA* **90,** 3899-3903.

Wang, B. B., Muller, I. M., Austin, J., Robinson, N. T., Chisholm, A. and Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. *Cell* **74,** 29-42.

Wassarman, D. A., Therrien, M. and Rubin, G. M. (1995). The Ras signaling pathway in *Drosophila*. *Curr. Opin. Genet. Dev*. **5,** 44-50.

Wood, W. B. (1988). In *The Nematode Caenorhabditis elegans*, (ed. W. B. Wood), New York: Cold Spring Harbor Laboratory.

Wu, Y. and Han, M. (1994). Suppression of activated Let-60 ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev*. **8,** 147-159.

Yochem, J., Weston, K. and Greenwald, I. (1988). The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila* Notch. *Nature* **335,** 547-550.