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Hox gene expression in a single *Caenorhabditis elegans* cell is regulated by a *caudal* homolog and intercellular signals that inhibit *Wnt* signaling

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SUMMARY

In *Caenorhabditis elegans* males, a row of epidermal precursor cells called seam cells generates a pattern of cuticular alae in anterior body regions and neural sensilla called rays in the posterior. The Hox gene *mab-5* is required for two posterior seam cells, V5 and V6, to generate rays. In *mab-5* mutant males, V5 and V6 do not generate sensory ray lineages but instead generate lineages that lead to alae. Here we show that two independent regulatory pathways can activate *mab-5* expression in the V cells. First, the *caudal* homolog *pal-1* turns on *mab-5* in V6 during embryogenesis. Second, a *Wnt* signaling pathway is capable of activating *mab-5* in the V cells during postembryonic development; however, during normal development Wnt signaling is inhibited by signals from neighboring V cells. The inhibition of this Wnt signaling pathway by lateral signals between the V cells limits the number of rays in the animal and also determines the position of the boundary between alae and rays.

Key words: *pal-1*, *mab-5*, Hox gene, *Wnt* pathway, *Caenorhabditis elegans*

INTRODUCTION

Intercellular signals and conserved Hox genes play an important role in patterning the peripheral nervous system of *C. elegans*. In this organism, a pattern of cuticular structures and neural sensilla arise from lateral rows of epidermal cells, called seam cells, which extend along both sides of the animal from head to tail (Sulston, 1988). Six seam cells, named V1-V6, lie along each side of the animal, and a seventh seam cell, T, is located posterior to the anus in the tail. Soon after hatching, the V cells begin to undergo rounds of classical stem-cell-like divisions, each cell generating a copy of itself and a second cell that becomes either an epidermal cell that fuses with the surrounding epidermal syncytium, a neuroblast, or another seam cell (Fig. 1). The seam cells ultimately generate cuticular ridges called alae, which extend along the two sides of the animal. Two types of sensilla are produced by the V cells: In both sexes, one of the descendants of the V5 cell, V5.pa, becomes a neuroblast that generates a postdeirid sensillum. In addition, in males, the descendants of V5.pp and V6 generate sensory rays instead of alae: the V5 lineage generates one ray and the V6 lineage generates five rays.

The boundary between cells that produce rays and cells that produce alae is established, at least in part, by a mechanism that requires intercellular signaling (Sulston and White, 1980; Waring and Kenyon 1990, 1991; Austin and Kenyon, 1994). If the posterior V5 and V6 cells are killed with a laser microbeam, then neighboring anterior V cells will produce ray neuroblasts. In addition, if V cells either anterior or posterior to V5 are killed, the V5 cell fails to produce the postdeirid neuroblast and produces additional rays. These changes in cell fate have been shown to be triggered by the absence of cell-cell contact (Waring and Kenyon, 1990, 1991; Waring et al., 1992; Austin and Kenyon, 1994). Prior to each round of cell division, the V cells are in contact with both an anterior and a posterior seam cell neighbor. After the V cells divide, their posterior daughters (the new seam cells) extend long, thin processes anteriorly and posteriorly across their sisters, the intervening presyncytial cells. These processes grow toward one another and make contact at about 8 hours after hatching (Austin and Kenyon, 1994). If the anterior or posterior neighbors of V5 (V2, V3 and V4 or else V6) are killed with a laser microbeam at hatching, the processes from the remaining seam cells do not make contact until much later, if at all. Under these conditions, the fate of the V5.pa cell changes: it does not become a postdeirid neuroblast, but instead becomes a seam cell. Ablation of neighboring cells changes the fate of V5.pa as long as the ablation is performed before 8 hours after hatching. Since this is the time at which the Vn.p processes make contact, this finding suggests that the signaling event required for cell fate commitment occurs when these cells make contact with one another. This interpretation is strengthened by the finding that treatments that delay the time of contact also delay the time at which commitment occurs (Austin and Kenyon, 1994).

The mechanism by which intercellular signaling effects a

change in the V5 and V6 lineages is not known; however, several findings suggest that the Hox gene *mab-5* may be involved. *mab-5* is required for the generation of many posterior-specific pattern elements, including the V5- and V6-derived rays, but it is not required for postdeirid formation (Kenyon, 1986). In wild-type animals, *mab-5* is ON in V6 at hatching, but OFF in the V5 lineage until much later, when it is switched on in the sister of the postdeirid, V5.pp, a seam cell that in males will produce a ray (Fig. 1; Salser and Kenyon, 1996). Two experiments suggest the hypothesis that *mab-5* is involved in the ablation-induced transformation of the postdeirid neuroblast into a seam cell: first, in *mab-5* null mutants, the frequency at which the transformation occurs is reduced (Austin and Kenyon, 1994), and second, expression of heat-shock *mab-5* early in postembryonic development mimics the effect of cell ablation and causes the V5.pa cell to become a seam cell instead of a postdeirid neuroblast (Salser and Kenyon, 1996).

Generation of the ray sensilla, like the postdeirid, is also sensitive to signaling between cells in the V lineages. If V6 is killed at hatching, then V5 generates a lineage similar to that of V6 and makes five rays (Sulston and White, 1980). As with postdeirid formation, it is possible that changes in *mab-5* expression are required to elicit the cell fate transformation; in *mab-5(–)* animals neither V5 nor V6 generate rays and administering *hs-mab-5* mimics the effect of ablating V6, causing V5 to generate a V6-like lineage and produce up to five rays (Salser and Kenyon, 1996).

The finding that killing the neighbors of V5 causes it to generate additional rays indicates that neighbor cells send inhibitory signals. This poses an interesting question: how is it that V6 is able to generate rays in the presence of these signals during normal development? Two possibilities are (i) the neighbors of V6 do not produce signals that inhibit ray formation, or (ii) V6 is immune to signals from neighboring cells. Analysis of the gene *pal-1* has suggested that model (ii) is correct (Waring and Kenyon, 1990, 1991). *pal-1* is the *C. elegans caudal* homolog and it plays an important role in patterning the posterior body region during early embryonic development (Hunter and Kenyon, 1996; L. G. Edgar and W. B. Wood, personal communication). In animals carrying a rare allele of *pal-1, e2091*, embryonic development is generally normal; however, V6 does not produce rays. Instead, V6 generates alae-producing lineages similar to those produced by V(1-4) (Fig. 1). However, if the seam cell neighbors of V6, that is either V4 and V5 or T, are killed with a laser microbeam in this mutant, then V6 generates its normal wild-type lineage and produces five rays (Fig. 1). *pal-1* has been shown to act in a cell-autonomous fashion to specify the wild-type V6 lineage. Therefore, it appears that the neighbors of V6 do send ray-inhibiting signals to V6 during development, but that the presence of *pal-1* activity in the V6 lineage allows the cells to ignore these signals and produce rays.

Although these studies indicate that *pal-1* activity allows the V6 cell to generate its normal lineage in spite of signals from its neighbors, they do not distinguish between two very different models for the role of *pal-1* in V6 development. One model is that *pal-1* promotes ray formation by acting specifically to block the effect of inhibitory signals; for example, by inhibiting the synthesis of a protein required for V6 to respond to signals from its neighbors, such as a receptor. This model predicts that the same gene products initiate ray formation in V6 both during normal development and following ablation. The second model is that *pal-1* circumvents the inhibitory signals; for example, by acting in a bypass pathway to initiate ray formation. In this case, one set of gene products initiates ray formation in V6 during normal development and a different set initiates ray formation following ablation of neighboring cells.

In this study, we show that both intercellular signals and *pal-1* activity regulate ray formation by controlling *mab-5* gene expression. In addition, we have carried out a series of experiments to distinguish between the two models for the role of *pal-1* in V cell development. Our findings favor the second model; namely that *pal-1* activity does not regulate signal transduction directly, but instead simply bypasses the effect of intercellular signals and activates *mab-5* by a signal-independent mechanism. We find that *pal-1* acts during embryogenesis to activate *mab-5* expression in V6, and that a separate pathway, a *Wnt* signaling pathway, can act later to activate *mab-5* expression following cell ablation. By repressing the activity of this *Wnt* pathway during normal development, signals between V cells play a key role in establishing a precise pattern of postdeirid and ray sensilla in this animal.

MATERIALS AND METHODS

Strains

Standard genetic procedures and growth conditions are described in Brenner (1974). The following strains were used: N2 wild-type var. Bristol, CF52: *pal-1(e2091) III; him-5(e1490)V,* CF142*: egl-20(mu25) IV,* CF383*: pal-1(ct224) dpy-17(e164) ncl-1(e1865) unc-36(e251) III; him-8(e1489) IV; sDp3(III;f),* CF401*: pal-1(e2091) III; egl-20 (n585) IV; him-5(e1490),* CF417*: lin-17(n671) I; him-5 (e1490) V,* CF451*: muIs16 [mab-5::gfp, pMH86] I; pal-1(e2091) III; egl-20 (n585) IV; him-5(e1490),* CF453*: muIs16 I; dpy-20(e1282)IV; him-5(e1490) V,* CF495*: muIs16 I; pal-1(e2091) III; him-5(e1490) V,* CF497*: muIs14 [mab-5::lacZ, rol-6(su1006d)] (*linkage unknown), HC3*: (muIs16) I; egl-20(n585) IV; him-5(e1490) V,* HC7*: lin-17(n3091) (muIs16) I,* HC9*: egl-20 (n585) IV; him-5(e1490) V,* HC10*: pal-1(e2091) III; muIs14.* CF491*: pry-1(mu38)I; him-5(e1490)V* CF731*: bar-1(ga80),* CF1072*: bar-1(ga80); him-5(e1490)*

Mosaic analysis

Ncl-1 mosaics from CF383 were identified directly by Nomarski differential interference contrast (DIC) microscopy by screening L3 larvae for mixtures of Ncl and non-Ncl cells (Herman, 1995). The origins of the Ncl clones were assigned by scoring closely related Ncl and non-Ncl cells. One mosaic was identified as a Pal-1 young adult male. This animal was Pal (that is, it expressed the V6 ray-to-alae transformation) on only one side. The origin of the Ncl clone was consistent with the phenotype.

Construction, integration and scoring of reporter gene constructs

Approximately 10 kb of the *mab-5* promoter region from a *Hin*dIII site to an artificial *Sma*I site in the 5′UTR (43 bp from the SL1 addition site) was fused to the *Sma*I site of TU#61 (Chalfie et al., 1994) creating plasmid pCH22, a *mab-5::gfp* reporter construct. A transgenic line expressing this construct was generated by co-injecting pCH22 with pMH86 [*dpy-20*(+)] (Han and Sternberg, 1990) into *dpy-20(e1282)* hermaphrodites. The *muIs16* integrated array was isolated following gamma irradiation (3800 rads). *dpy-20(e1282)* was not

maintained in subsequent out-crosses and strain constructions. GFP localization patterns are similar to that described for a *lacZ* reporter (Cowing and Kenyon, 1992) and MAB-5 antibody staining (Salser et al., 1993; Salser and Kenyon, 1996). However, similar to *lacZ* reporters, expression levels in non-neuronal cells begin receding during late L1 and early L2 stages.

The *mab-5::lacZ* reporter used in this analysis is similar to previously described constructs except an additional 2.7 kb of promoter to a *Hin*dIII site was added (10 kb total). This construct, pCH7, was coinjected with pRF4 [*rol-6(su1006d)*] (Mello et al., 1991) and a spontaneous integrant *muIs14* was isolated. β-GAL expression was detected using a monoclonal antibody specific for β-GAL (Promega) and rhodamine-conjugated secondary antibodies (Jackson labs). The β-GAL expression pattern produced by this construct was similar to that previously described for *mab-5::lacZ* reporters (Cowing and Kenyon, 1992), except expression in the eight ABp(l/r)ppppxxx cells was not detected. *mab-5* is not required for the development of the descendants of these cells. Thus, this result suggest that sequences in the additional 2.7 kb inhibit expression in these cells, although the site of integration may also affect the expression pattern.

Immunolocalization of PAL-1

Immunolocalization of PAL-1 was as previously described (Hunter and Kenyon, 1996). For all antibody staining results reported here, staged embryos (1- to 4-cell) were incubated for 4 hours at 25°C and then fixed for staining.

Photomicrographs were recorded on Ektachrome 400 film and digitized using a Kodak RFS 2035 film scanner. The PAL-1, β-GAL double staining was scored and recorded on a Zeiss confocal microscope.

Cell ablation

For cell ablation experiments, all animals were synchronized by hatching (30 minute window) and cells were ablated within an equivalent interval. Ablations were performed as described (Austin and Kenyon, 1994). Control animals were treated identically to experimental animals and often included the non-ablated side of experimental animal (as noted in the text). To monitor GFP expression in seam cells following cell ablation, animals were checked briefly, at most every 2 hours, to minimize photo damage.

RESULTS

Signals from neighboring V cells regulate the expression of the Hox gene *mab-5*

A number of observations suggest that the V cell fate transformations that occur following ablations of neighboring cells might be caused by inappropriate expression of *mab-5* activity. All of the V cell fate transformations that occur following ablations of neighboring cells require *mab-5* activity (Waring and Kenyon 1990; Austin and Kenyon, 1994). Furthermore, ectopic expression of *mab-5* mimics the effect of ablation: V5.pa generates a seam cell instead of a postdeirid, and it generates additional rays (Salser and Kenyon, 1996). *mab-5* activity is also required to allow V6 to generate rays after the ablation of T in a *pal-1* mutant (Waring and Kenyon, 1990).

During wild-type development, *mab-5* is not expressed in the V5 lineage until after the time that the postdeirid neuroblast has been born. In addition, it is only expressed in the most posterior branch of the V5 lineage, in which ray specification occurs (see Fig. 1). To ask whether ablation of neighboring cells might cause V5 to express *mab-5*, we asked whether expression of a *mab-5*-GFP fusion could be switched on precociously in the V5 lineage following ablation of V6. We found that following ablation of V6, GFP expression was detected in V5.p and both seam cell daughters (Fig. 2). We verified this finding using anti-MAB-5 antisera (S. Salser and C. K., unpublished data). This finding, together with the genetic analysis of *mab-5*, suggests that the reason the V5.pa fate is changed following ablation of neighboring cells is because ablation of neighboring cells causes *mab-5* to be expressed, which, in turn, causes V5.pa to generate a seam cell instead of a postdeirid neuroblast. This ectopic expression of *mab-5* can also explain why V5 generates additional rays in males.

Can changes in *mab-5* expression also explain the ability of V6 to generate its wild-type lineage following ablation of neighboring cells in a *pal-1(e2091)* mutant? In *pal-1(e2091)* mutants, *mab-5* is not expressed in V6 at hatching (Salser and Kenyon, 1996). Moreover, expression of *mab-5* using either a gain-of-function *mab-5* mutant *(e1751)* (Waring and Kenyon, 1991) or a *hs-mab-5* fusion (C. P. H. and C. K., unpublished data) can suppress the Pal-1(e2091) mutant phenotype and allow V6 to generate rays. To test the hypothesis that ablation

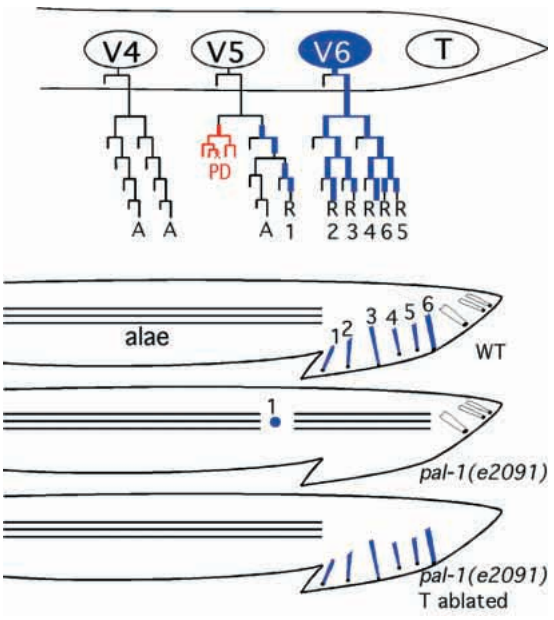

Fig. 1. Schematic of MAB-5 expression pattern and male tail phenotypes. MAB-5 (blue) is expressed in the V6 seam cell at hatching and continues to be expressed in all dividing V6 descendants (Salser and Kenyon, 1996). In the V5 seam cell lineage, MAB-5 is first detected in the V5.pp cell (blue lines) late in the cell cycle. MAB-5 expression switches on and off several times in this lineage. The postdeirid neuroblast (V5.pa) and the lineage of its descendants are shown in red. In wild-type adults, six *mab-5*-dependent ray sensilla (blue) are produced by V5 and V6; the T seam cell also produces three rays (white). In *pal-1(e2091)* animals, the rays from V6 are missing and the seam cells generate lineages similar to those of V1-V4 and produce alae. As a consequence, ray 1 is displaced anteriorly in the body in a gap between the alae (Waring and Kenyon, 1990). Ablation of the T seam cell suppresses the Pal-1 phenotype, thereby restoring the production of all *mab-5*-dependent rays.

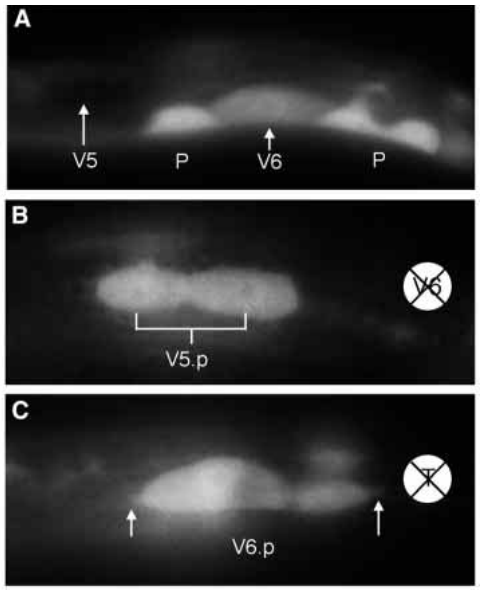

Fig. 2. *mab-5::*GFP expression in seam cells. (A) In wild-type L1 larvae, *mab-5::*GFP was detected in V6 and surrounding P cells but not in V5. Residual GFP in hyp7, presumably from C descendants that express *mab-5* during embryogenesis, often outlines the non-expressing V5 seam cell. (B) Ablation of V6 resulted in *mab-5::*GFP expression in V5.p and its descendants. In this example V5.p has just divided. (C) In *pal-1(e2091)* mutants, *mab-5::*GFP was not detected in V6 or its descendants (not shown) but was expressed strongly in V6.p following ablation of the T seam cell. The white arrows point to the extending V6.p processes.

of neighboring cells rescues the Pal-1 mutant phenotype by allowing V6 to switch on expression of *mab-5*, we ablated T at hatching in a *pal-1(e2091)* mutant and asked whether this caused cells in the V6 lineage to switch on expression of *mab-5*-GFP. We found that it did (Fig. 2). Thus we conclude that the reason that these ablations rescue the Pal-1 mutant phenotype is that they allow V6 to turn on *mab-5*.

PAL-1 protein acts within V6 to turn on *mab-5* expression during embryogenesis

The experiments described above show that, both in wild-type animals and in *pal-1* mutants, cell signals normally inhibit *mab-5* expression, and that cell ablations prevent postdeirid formation and trigger ray formation by turning on *mab-5* gene expression. Given this, the key question becomes: how is it that V6 is able to remain immune to signals from neighboring cells and generate rays during normal development? Since this immunity disappears in animals carrying a *pal-1(e2091)* mutation, the answer seems likely to lie with the *pal-1* gene.

The *pal-1* gene is known to have both maternal and zygotic roles in early embryonic development (Hunter and Kenyon, 1996; L. G. Edgar and W. B. Wood, personal communication). As described above, the *pal-1(e2091)* mutation does not affect embryonic development, but instead affects the ability of V6 to adopt its normal fate and generate rays rather than alae during postembryonic development. This phenotype, plus the fact that this allele is recessive, suggest that this *pal-1* mutation reduces *pal-1* activity in the V6 lineage (see Waring and Kenyon, 1990).

Since the *pal-1(e2091)* mutation is an unusual allele, we first confirmed that its phenotype resulted from lack of *pal-1* activity and not from a novel activity. To do this, we isolated *pal-1* genetic mosaics using a strong loss-of-function allele, *ct224*. *ct224* is an approximately 4 kb deletion that removes most of the *pal-1* coding region and is recessive lethal (L. G. Edgar and W. B. Wood, personal communication; Yandell et al., 1993). We isolated viable *pal-1(ct224)* genetic mosaics using the linked mutation *ncl-1* as a genotypic marker (Fig. 3). Among these mosaic animals were two males with Pal phenotypes; in both cases, the V6 cell was Ncl and therefore mutant for *pal-1*. This indicated that this V6 ray phenotype was indeed due to lack of *pal-1* activity.

We also identified several other Ncl-1 mosaic males in which other cells that require *mab-5* activity were Ncl. Four of these animals were *pal-1(–)* in ABp-derived cells and produced animals that appeared wild type. Since ABp gives rise to V5, this finding indicates that cells in the V5 lineage do not require *pal-1* activity to develop normally. In addition, we observed that other *mab-5*-dependent pattern elements derived from ABp, such as the hook and the migratory pattern of the QL descendants, were all normal. Finally, one mosaic male was Ncl only in MSa descendants and had crumpled spicules, a phenotype consistent with loss of *mab-5* function in the MSa-derived sex myoblast, M (Kenyon, 1986). This suggests that *pal-1* function is required in M for *mab-5* function. Together these findings suggest that *pal-1* function is required in M as well as V6 for *mab-5*-dependent functions, but not for the majority of *mab-5*-dependent cell fates.

The experiments described above indicate that *pal-1* mutants do not generate V6 rays because V6 does not express *mab-5*. *mab-5* expression in V6 is known to be initiated during mid-

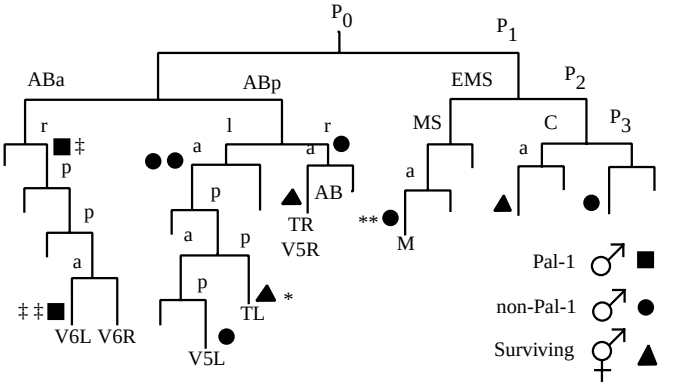

Fig. 3. Embryonic lineage of *mab-5*-expressing cells showing origins of surviving *pal-1(ct224)* clones. Each symbol represents a single mosaic animal and the placement of the symbol marks the point of duplication loss as determined by the Ncl-1 enlarged nucleoli phenotype. *Hermaphrodite had ventral bulge characteristic of some animals carrying the *pal-1(e2091)* allele. **Male had crumpled spicules but was otherwise wild type. The same crumpled spicule phenotype is seen in Mab-5(–) mutants and is caused by incorrect migration of the sex myoblasts. This suggests that *pal-1* function is required in M for *mab-5* function. Consistent with this interpretation, PAL-1 protein was detected in the M cell during embryogenesis before MAB-5 expression in M is initiated (L. G. Edgar and W. B. Wood, personal communication; our unpublished observations). ‡V6 rays were transformed to alae on both sides (Pal-1 phenotype). ‡‡V6 rays were transformed to alae on the left side only.

Table 1. The *pal-1(e2091)* mutation prevents PAL-1 accumulation and also *mab-5::lacZ* expression in posterior epidermal cells

	mab-5::lacZ		*pal-1(e2091); mab-5::lacZ*	
Cells examined	β-GAL	PAL-1	β-GAL	PAL-1
P 9/10	15/15	14/15	46/53	1/53
P11/12	15/15	0/15	53/53	0/53
V6	15/15	15/15	8/53*	0/53
C(a/p)aapp	8/15	7/15	8/53	1/53

*The β-GAL detected in these V6 cells was barely detectable.

embryogenesis (Cowing and Kenyon, 1992). Thus we wanted to know whether *pal-1* was needed to turn on *mab-5* at this time. We first asked whether PAL-1 is expressed in V6 at the time of *mab-5* activation. To do this, we stained *mab-5::lacZ*-expressing embryos with both anti-β-GAL antibodies to identify *mab-5*-expressing cells and with anti-PAL-1 antibodies (Hunter and Kenyon, 1996). We detected both PAL-1 and β-GAL in V6, as well as P9/10, and C(a/p)aapp (Fig. 4A; Table 1). Thus, PAL-1 is present in V6 when *mab-5* expression is initiated.

Why is *mab-5* not expressed in V6 in *pal-1(e2091)* mutants? To test the possibility that PAL-1 protein was not present in V6 in this mutant, we examined its pattern of PAL-1 protein using anti-PAL-1 antibodies. To monitor *mab-5* expression at the same time, we also monitored *mab-5::lacZ* expression using anti β-GAL antibodies. We found that neither PAL-1 nor β-GAL were detected in V6 at this early time (Fig. 4B; Table 1). In addition, PAL-1 was rarely detectable in P9/10 or the C descendants; however, PAL-1 was still detectable in many posterior nuclei. This suggests that either PAL-1 is not expressed in these cells in *e2091* mutants or that the *e2091* gene product is unstable in these cells. We favor the former interpretation because, when we sequenced *pal-1* genomic DNA corresponding to the exons and intron/exon boundaries, we failed to detect any sequence changes. These results lead us to believe that *e2091* is a regulatory mutation that prevents PAL-1 expression in a subset of its normal domain of function and that lack of PAL-1 in V6 results in the lack of *mab-5* expression in V6 (Fig. 6A,B).

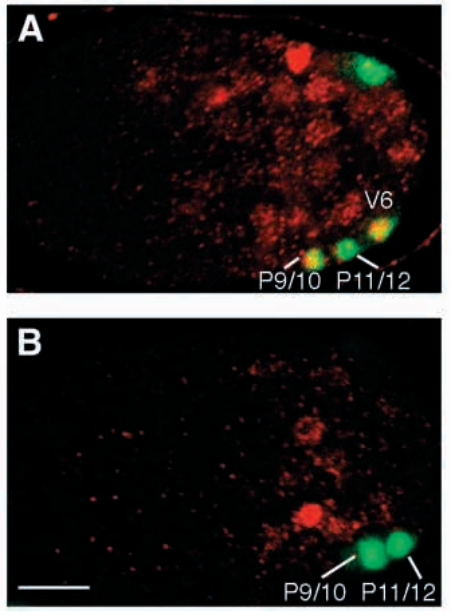

Fig. 4. *mab-5::lacZ* expression and PAL-1 localization in wild-type and *pal-1(e2091)* embryos. Anti-β-GAL (green) and anti-PAL-1(red) immunofluorescent confocal-micrograph of (A) wild-type and (B) *pal-1(e2091)* embryos expressing integrated *mab-5::lacZ* reporter gene *muIs14*. (A) Both PAL-1 and *mab-5::lacZ* were expressed in wild-type P 9/10 and V6 cells (yellow) whereas P11/12 expressed *mab-5::lacZ* only. (B) PAL-1 was not detectable in either P 9/10 or V6 in *pal-1(e2091)* mutants and *mab-5::lacZ* was not expressed in V6. *mab-5::lacZ* was still expressed in P9/10 in most animals (see Table 1 for quantitation). Scale bar: 5 μm.

PAL-1 and the post-ablation *mab-5* activation pathway act at different times, and thus must be distinct from one another

The experiments described above indicated that PAL-1 protein turns on *mab-5* expression in the embryo. We next investigated

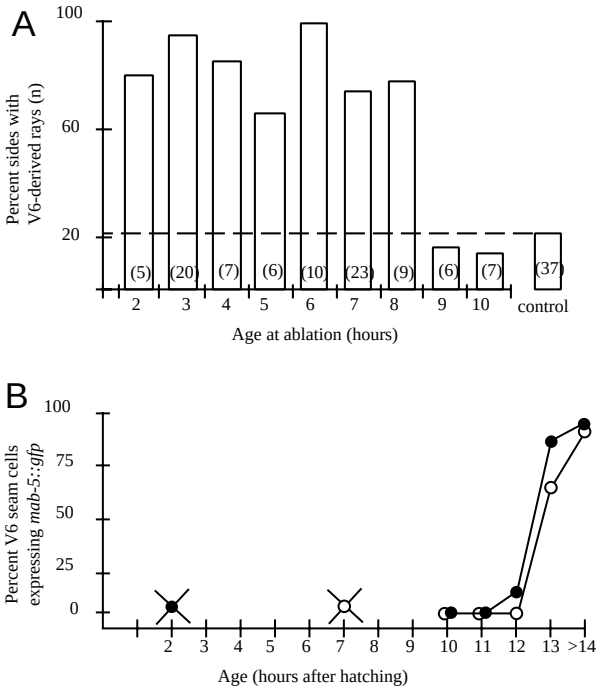

Fig. 5. Time courses of *pal-1(e2091)* suppression by T-cell ablation and *mab-5::*GFP expression following T-cell ablation. (A) The T seam cell or its descendants (T, T.a or T.ap) was ablated at the indicated times after hatching. Early ablations efficiently suppressed the Pal-1 phenotype (scored in the adult) while later ablations did not. Control sides were scored on non-ablated sides and non-ablated animals on the same slides as ablated animals. The number of animals examined at each time is shown in parentheses. (B) The T cell (filled circle) was ablated at 2 hours or T.ap (open circle) was ablated at 7 hours and expression of *mab-5::*GFP in V6.p was monitored periodically (see Materials and Methods). V6.p cells on the non-ablated sides of experimental animals never expressed *mab-5::*GFP. Number of V6.p cells scored for GFP for T cells ablated at 2 hours n=12 (10 hours), n=10 (11 hours), n=6 (12 hours), n=7 (13 hours) and n=15 (14 and greater hours) and for T.ap cells ablated at 7 hours n= 7 (10 hours), n=6 (11 hours), n=12 (12 hours), n=6 (13 hours), n=12 (12 hours).

Table 2. Effect of Wnt pathway mutations on fates of V5 descendants following ablation of the V6 cell

A

Genotype	V6 ablated?	V5.pa fate: postdeirid neuroblast	V5.pa fate: mixed lineage	V5.pa fate: seam cell	(*n*)	*mab-5*::gfp in V5.pa (*n*)
WT	no	100%	0%	0%	(29)	
WT	yes	0%	0%	100%	(10)	
muIs16	no	100%	0%	0%	(10)	0% (10)
muIs16	yes	0%	0%	100%	(7)	100% (5)
egl-20(n585)	yes	32%	12%	56%	(25)	
egl-20(mu25)	yes	67%	17%	17%	(6)	
muIs16; egl-20(n585)	yes	80%	0%	20%	(5)	0% (6)
lin-17(n671)	yes	33%	0%	67%	(6)	
lin-17(n3091)	yes	33%	17%	50%	(6)	
muIs16; lin-17(n3091)	yes	43%	43%	17%	(7)	0% (7)
bar-1(ga80)	yes	88%	0%	13%	(8)	
*mab-5(e1239)**	yes	60%	30%	10%	(20)	

B

Genotype	Number of V rays in the male tail: 1	2	3	4	5	(*n*)
him-5(e1490)	0	18%	12%	6%	65%	(17)
egl-20(n585); him-5(e1490)	11%	78%	6%	0	6%	(18)
bar-1(ga80); him-5(e1490)	70%	10%	10%	0	10%	(10)

*From Austin and Kenyon (1994).

the mechanism by which cell ablations activated *mab-5* expression. Previous studies had shown that, in order to effect the V5 cell fate transformation triggered by ablation of neighboring cells, the ablation had to be carried out before the time that the processes extending from the Vn.p seam cells touched one another, approximately 8 hours after hatching. As long as the ablation was carried out before this time, V5.pa generated a seam cell; if ablation was carried out after this time, it generated its normal postdeirid. To determine whether the V6 cell fate transformation triggered by cell ablation in *pal-1(e2091)* mutants was also sensitive to this time constraint, we ablated the T seam cell at successively later times after hatching to determine when it was no longer possible to suppress the *pal-1* phenotype. We found that ablating the T seam cell up to and including the 8 hour time point reproducibly suppressed the Pal-1 phenotype in V6, but that ablation at 9 and 10 hours did not (Fig. 5A). This is the same developmental time at which signals between V5 and its neighbors were found to act (Austin and Kenyon, 1994). These findings suggest that the mechanism for the cell-ablation-induced cell fate transformations of V5 and V6 are similar.

Since the change in V cell fate appears to be caused by expression of *mab-5*, we were curious to know when after cell ablation *mab-5* would be expressed. One can imagine two possibilities: first, it is possible that *mab-5* would always be expressed quite soon after ablation, no matter when the ablation was carried out. Such a finding would suggest that the ablation itself directly set in motion a cascade of events that led to *mab-5* activation. Alternatively, it was also possible that, after neighbor ablation, there would be a discrete time point in which *mab-5* would be switched on and, moreover, that this time point would be independent of the time at which the ablation was carried out. This finding would indicate that ablation would not directly trigger *mab-5* activation, but rather that it would pave the way for a discrete *mab-5*-activation pathway to act at a later time during development. We carried out two experiments to determine the time course of *mab-5* expression following ablation of neighboring cells. First, we ablated the T seam cell 2 hours after hatching and monitored V6 and V6.p in *pal-1(e2091)* mutants for *mab-5*::GFP expression. We first detected *mab-5*::GFP after a considerable lag period, at 12 hours after hatching in the V6.p seam cell (Fig. 5B). We then ablated the seam cell T.ap in *pal-1(e2091)* at 7 hours after hatching, just before cell ablations are no longer effective. Again, we first detected *mab-5*::GFP at 12 hours (Fig. 5B). Thus the time at which *mab-5* was switched on correlated with developmental stage, rather than the time of ablation. These experiments suggest that there is a *mab-5*-activation pathway that has the potential to switch on *mab-5* at 12 hours after hatching, but only if neighboring seam cells have been ablated.

The pathway that activates *mab-5* following cell ablations is a *Wnt* signaling pathway

Since our findings suggested that, in addition to *pal-1*, there exists a second *mab-5* activation pathway that can activate expression of *mab-5* at 12 hours after hatching, we asked what genes might constitute the pathway. To do this, we examined animals carrying mutations known to prevent *mab-5* expression in other cells. One key *mab-5* activation pathway is known to be a *Wnt* signaling pathway, which activates *mab-5* expression in QL, a migratory neuroblast, during normal development (Harris et al., 1996; Maloof et al., 1999). This pathway comprises a *Wnt* homolog, encoded by *egl-20* (Maloof et al., 1999), a putative receptor, a *frizzled* homolog encoded by *lin-17* (Sawa et al., 1996), and an *Armadillo* homolog encoded by *bar-1* (Eisenmann et al., 1998). This pathway also contains a negative regulator, encoded by the *pry-1* gene, which inhibits *bar-1* activity in the absence of *egl-20/Wnt* activity. In *egl-20/Wnt*, *lin-17/frizzled* and *bar-1/Armadillo* mutants, *mab-5* is not expressed in QL. In *pry-1* mutants, *mab-5* is expressed in QL independently of *egl-20*,

Table 3. Effect of *egl-20* mutations on the number of V rays and *mab-5::*GFP expression in V6.p following T cell ablation

Genotype‡	T ablated?	Number of V rays				*mab-5::*GFP in V6.p % (*n*)
		0-1	2-3	4-5	(*n*)	
pal-1	no	88%	8%	4%	(49)	
pal-1	yes	11%	11%	78%	(37)	
muIs16; pal-1	no	100%	–	–	(8)	0% (19)
muIs16; pal-1	yes	23%	15%	62%	(13)	100% (19)
pal-1; egl-20	no	95%	–	5%	(19)	
pal-1; egl-20	yes	80%	15%	5%	(20)	
muIs16; pal-1; egl-20	no	100%	–	–	(10)	0% (26)
muIs16; pal-1; egl-20	yes	100%	–	–	(10)	0% (14)
egl-20	no	–	–	100%	(11)	
egl-20	yes	–	–	100%	(9)	

‡All strains also contained the *him-5(e1490)* mutation to increase the number of self-progeny males. The *pal-1(e2091)* and *egl-20(n585)* alleles were used for all strains.

but not *bar-1* (Maloof et al., 1999). In this mutant, *mab-5* is also expressed in QL's bilateral homolog QR, which normally does not express *mab-5*, and also in many other cell types. Most importantly, in *pry-1* mutants, *mab-5* is expressed precociously in the V5 lineage and V5 does not make a postdeirid and generates additional rays, just as it does after neighbor ablation (Maloof et al., 1999).

To ask whether this *Wnt* signaling pathway might be the post-ablation *mab-5*-activation pathway in the V cell lineages, we first asked whether *egl-20, lin-17* and *bar-1* mutants prevented the postdeirid-to-seam cell fate transformation that normally takes place in the V5 lineage following ablation of V6. We ablated V6 and scored for postdeirid formation in *egl-20, lin-17* and *bar-1* mutants. We found that all three genes were required for efficient transformation of V5.pa from a neuroblast to a seam cell (Table 2A). We also scored V6-ablated *egl-20* and *bar-1* mutant males for the V5-derived ray neuroblast. In wild-type males, V5 often produces five ray neuroblasts following V6 ablation. We found that, in both *egl-20* and *bar-1* mutants, V5 most often produced fewer ray neuroblasts following V6 ablation (Table 2B). (We were unable to carry out these ablations in *lin-17* mutants because of the poor health of these animals).

The finding that mutations in these *Wnt* pathway genes prevented cells from responding to ablation of their neighbors suggested that *egl-20, lin-17* and *bar-1* were required to activate *mab-5* following cell ablation. To test this hypothesis, we ablated V6 in *mab-5*::GFP strains containing either *egl-20* or *lin-17* mutations. We found that GFP was never detected in V5.pp or V5.pa in these strains (Table 2A). We conclude that the *Wnt* signaling pathway is required for V5 to express *mab-5* following ablation of V6.

We also asked whether the *Wnt* pathway is required in order for V6 to express *mab-5* in a *pal-1(e2091)* mutant following neighbor ablation. Mutations in *egl-20, lin-17* and *bar-1* do not normally affect *mab-5* expression or *mab-5* function in V6 (Harris et al., 1996; Maloof et al., 1999); however, our findings that these genes activate *mab-5* in V5 following ablation suggested that they might also activate *mab-5* in V6.p in *pal-1*(*e2091*) mutants following T seam cell ablation. To test this idea, we ablated T in *pal-1(e2091); egl-20* double mutants and found that no V rays were produced (Table 3). This indicates that *egl-20* function is required in order for T cell ablation to suppress *pal-1(e2091)*. We next investigated whether *egl-20/Wnt* function is required to activate *mab-5*::GFP expression in V6.p following ablation of T. When we ablated T in *pal-1; egl-20* double mutants, we never detected GFP expression in V6.p or in V6.p(a/p) (Table 3). Therefore, *egl-20/Wnt* function is required in order for *pal-1(e2091)* mutant V6-derived cells to turn on *mab-5* when T is ablated. We attempted to analyze *lin-17; pal-1* double mutants, but these animals proved to be extremely sick and difficult to score.

The experiments described above suggest that intercellular signals block *mab-5* expression in V5 and V6 by inhibiting a *Wnt* pathway. Cell ablation seems to remove these inhibitory signals, thereby allowing the *Wnt* signaling pathway to activate *mab-5* expression. If this is the case, then constitutive *Wnt* pathway activity should bypass the inhibitory intercellular signals and activate *mab-5* expression. Thus, like cell ablation, constitutive activation of the *Wnt* pathway should suppress a *pal-1(e2091)* mutation. To test this prediction, we used *pry-1* mutants in which, as noted above, *bar-1/Arm* activates *mab-5* in a constitutive, *egl-20/Wnt*-independent fashion (Maloof et al., 1999). We found that a *pry-1(mu38); pal-1(e2091)* double mutant produced an average of 7.5 rays in the tail (*n*=98) compared to *pal-1(e2091)*, which produced an average of 3.5 rays in the tail (*n*=49). This observation supports the conclusion that a *Wnt* signaling pathway can regulate *mab-5* expression in V6 and that this pathway is normally inhibited by intercellular signals (Fig. 6C,D).

Lack of cell contact appears to trigger *mab-5* activation

Disruption of neighbor cell contact on either side of V5 is sufficient to inhibit postdeirid formation (Sulston and White, 1980; Austin and Kenyon, 1994). For example, ablation of V6 changes the fate of V5.p even though V5.p still contacts its anterior neighbor V4.p. Conversely, ablation of the anterior neighbors of V5 changes V5.p's fate even though V5.p still contacts its posterior neighbor V6.p. This surprising finding has suggested the possibility that loss of cell contact on one side of V5.p is 'dominant' to the presence of cell contact on the other, and thus that the signal that changes the fate of V5 is created by lack of cell contact. Alternatively, different mechanisms could be activated by anterior and posterior ablations. If the *Wnt* pathway genes are not required for signaling following anterior neighbor cell ablations, then anterior and posterior ablations act through distinct pathways. If the *Wnt* pathway genes are required, then neighbor cell ablations may act 'dominantly'. To distinguish between these

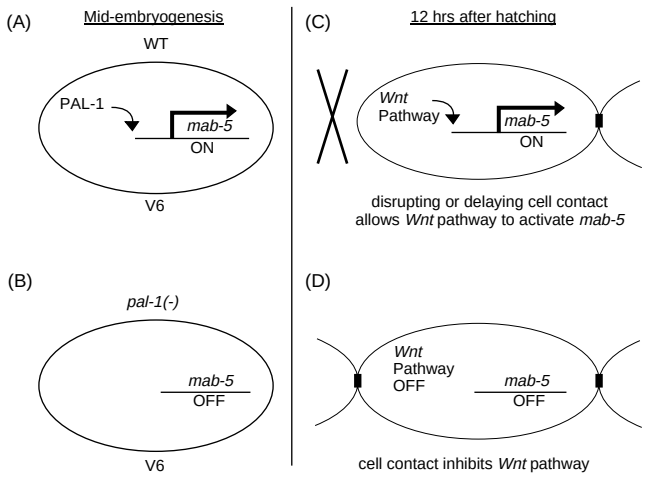

Fig. 6. Model for two different pathways that activate *mab-5* expression in V cells. (A) In wild-type embryos PAL-1 acts directly or indirectly to activate *mab-5* expression in V6. (B) In *pal-1(e2091)* mutants PAL-1 is not detected in V6 and *mab-5* is not expressed. (C) Cell ablation, by disrupting or delaying cell contact between V cells, allows an *egl-20 Wnt* pathway to activate *mab-5* expression in wild-type V5 cells and also in *pal-1(e2091)* V6 cells. (D) Cell contact inhibits *Wnt* pathway activity. *Wnt* signal transduction may take place in V5 and V6 (as shown) or may take place in neighboring cells.

two models, we ablated the anterior neighbor cells V2-V4 in both *egl-20* and *lin-17* mutants (Table 4). We found a stringent requirement for *egl-20* function, which favors the model that the unconnected process present on one side of V5.p creates a dominant signal that changes V5.p's fate. We found a weaker requirement for *lin-17* function. This may indicate that an additional *Frizzled* homolog functions along with *lin-17* to mediate signaling between V5.p and its anterior neighbors.

DISCUSSION

In this paper, we have investigated the mechanism by which intercellular signals influence the pattern of postdeirid and ray sensilla that are formed within the lateral epidermis of *C. elegans*. These signals allow the production of a postdeirid sensillum and prevent the production of additional sensory rays in the V5 cell lineage. In addition, we have asked how the homeobox gene *pal-1* functions within the V6 lineage to confer immunity to these signals.

Our findings indicate that the key regulatory point in this circuit is the expression of the Hox gene *mab-5*, which represses postdeirid formation and initiates ray formation. We find that the signals between cells in the lateral epidermal lineages prevent a *Wnt* signaling pathway from activating *mab-5* in both the V5 and V6 lineages. *pal-1* overcomes the effects of these signals in V6 not by counteracting them directly, but by activating *mab-5* by an independent mechanism (Fig. 6).

Table 4. Effect of *egl-20* and *lin-17* mutations on the fates of the V5 descendants following ablation of the anterior seam cells V2, V3 and V4

		V5.pa fate			
	V2, V3, V4 ablated?	postdeirid neuroblast	mixed lineage	seam cell	(*n*)
WT	no	100%	0%	0%	(29)
WT	yes	0%	0%	100%	(1)
egl-20(n585)	yes	57%	29%	14%	(7)
lin-17(n3091)	yes	9%	0%	91%	(11)
*mab-5(e1239)**	yes	35%	15%	50%	(20)

*From Austin and Kenyon (1994).

Signals from neighboring cells exert their effects by repressing expression of the Hox gene *mab-5*

In the wild type, *mab-5* expression is switched on in the embryo in the V6 lineage, but not until much later, during postembryonic development, in the V5.pp cell (Cowing and Kenyon, 1992; Salser and Kenyon, 1996). Expression of *mab-5* allows specific cells in the V6 and V5.pp lineages to produce ray sensilla during the final stages of postembryonic development in the male. Previous genetic experiments had suggested that the reason that the pattern of postdeirid and ray sensilla changes following ablation of neighboring cells might be that *mab-5* expression is activated (Austin and Kenyon, 1994). This is because loss of *mab-5* activity prevents the cell fate changes normally observed following ablation of neighboring cells, and because ectopic *mab-5* expression mimics the effect of these ablations. In this study, we have used reporter-gene fusions and anti-MAB-5 antisera to visualize *mab-5* expression in lineages whose neighbors have been ablated. As predicted by the hypothesis, we find that cell ablations trigger *mab-5* expression in these lineages. Thus the question of how intercellular signals affect pattern formation reduces to the question of how intercellular signals block *mab-5* expression.

Expression of *mab-5* following ablation of neighboring cells commences at a specific stage during postembryonic development

During wild-type development, *mab-5* expression within the V5 lineage begins in the V5.pp cell, late in the cell cycle. We found that, if neighboring cells were ablated, then *mab-5* expression began during the previous cell cycle, in the V5.p cell, or in the V6.p cell in *pal-1(e2091)* mutants. Surprisingly, we found that *mab-5* expression was turned on at this time no matter when the ablation was performed. This was an informative result for the following reason: one could imagine that all of the *mab-5* activation machinery was present in the V cells and was prevented from acting only because of *mab-5*-repressive signals from neighboring cells. If this were the case, then one would expect that *mab-5* expression would commence at a fixed interval following ablation of neighboring cells; the length of this interval would correspond to the time necessary for repressive signals to abate and the ever-ready activation machinery to begin operating. In contrast, the finding that *mab-5* expression did not begin until 12 hours after hatching irrespective of when the ablations were performed indicated that the post-ablation activation machinery was not operative until a later stage in postembryonic development. Apparently, ablation of neighboring cells removes an obstacle to the later operation of this activation pathway.

Two different pathways can activate Hox gene expression in the V6 lineage

The Pal-1(e2091) phenotype is particularly interesting because it can be completely overcome by cell ablation. Thus, an important goal of this study was to determine how *pal-1* and cell ablations promote ray development. In particular, we wished to determine whether the normal role of *pal-1* is to block the effect of inhibitory cell signals or whether *pal-1* acts more directly. The first significant finding was that *mab-5* expression is the target of both PAL-1 in the embryo and the effect of cell ablations after hatching. The second significant finding was that mutations in the *Wnt* pathway genes *egl-20*, *bar-1* and *lin-17* block *mab-5* activation following cell ablation, implying that cell contact inhibits *Wnt* signal transduction, but do not affect *mab-5* expression initiated during embryogenesis. This implies that *pal-1* does not function in the embryo to allow *Wnt*-mediated activation of *mab-5*. Therefore the simplest interpretation is that separate pathways act to initiate *mab-5* expression in the V6 lineage; *pal-1* in the embryo and the *Wnt* pathway following cell ablation (Fig. 6). Thus, the reason the Pal-1(e2091) phenotype can be suppressed by cell ablation is that *Wnt*-dependent activators compensate for the failure of *pal-1(e2091)* to activate *mab-5* expression in the embryo. It is even possible that PAL-1 may act directly to activate *mab-5* expression.

Direct activation of *mab-5* expression by PAL-1 is consistent with the action of *caudal* homologs in other species. Both *Drosophila cad* and mouse *cdx-1* activate Hox genes. *cdx-1* binds Hox promoter sequences and *cad* protein directly activates the pair-rule gene *ftz* (Subramanian et al., 1995; Dearolf et al., 1989). Furthermore, we have identified several consensus Caudal-binding sites in the *mab-5* promoter (Dearolf et al., 1989).

A *Wnt* signaling pathway can turn on *mab-5* expression in the V cells

By testing known *mab-5* activation mutants, we discovered that the second *mab-5* activation pathway, which can operate 12 hours after hatching, was a *Wnt* signaling pathway. Thus, two very different mechanisms exist to activate Hox gene expression in the same cell: *pal-1* during normal development and a *Wnt* signaling pathway following ablation of neighboring cells.

We have shown that contact between seam cells inhibits *Wnt* signal transduction. How do interactions between the seam cells inhibit *Wnt* signaling? Previous findings (Austin and Kenyon, 1994) suggest that the time at which inhibition of *Wnt* signaling becomes irreversible is the time that the processes extending from the Vn.p seam cells form connections with one another – about 8 hours after hatching. If this contact (or close approximation) is prevented, then *mab-5* expression will commence about 4 hours later (this work). Furthermore, the observation that ablation of neighbors on either side of a V cell is sufficient to initiate a fate transformation indicates that loss of cell contact may activate *Wnt* signaling. How might this occur? One possibility is that contact between neighboring seam cells prevents activation of *Wnt* receptors which could be localized to the tips of the V cell processes. For example, a *Wnt* inhibitor, such as FrzB (Leyns et al., 1997; Wang et al., 1997) could be secreted locally in response to cell contact, inhibiting anterior and posterior localized Frizzled receptors independently; failure to inhibit both would lead to *Wnt* pathway activity. Alternatively, the interactions between the seam cells could prevent signaling by influencing an intracellular component of the signaling pathway: for example, a seam cell process that does not contact a neighbor seam cell could sequester a *Wnt* pathway inhibitor. Why might the *C. elegans* V cells have evolved the potential to change their fates in response to the lack of signals from their neighbors? One possibility is that this system arose during evolution to ensure that developing nematodes produced the proper number of epidermal cells and sensilla. The consequence of ablation is that the remaining V cells undergo additional rounds of cell division, producing additional sensilla and also additional epidermal cells. This suggests that ablation of V cells may activate a growth-control mechanism in the remaining cells (Sulston and White, 1980; Austin and Kenyon, 1994). It is possible that this intercellular signaling system is used during the normal development of other nematode species to control cell growth. The number of V cells present at hatching in other, larger nematodes is the same as in *C. elegans* but these cells undergo extra, and variable, numbers of proliferative divisions during larval growth (Sternberg and Horvitz, 1982). If the density of epidermal cells is low enough that connection does not occur in a timely fashion, then the *Wnt* pathway would be activated, promoting additional rounds of cell division that lead to additional epidermal cells and body structures. Conversely, contact between proliferating seam cells would serve to inhibit *Wnt*-dependent growth and promote alternative pathways of differentiation that generate fewer cells. In this regard, it is interesting to note that since *mab-5* is required for the change in cell number that occurs following cell ablation, Hox genes may be directly involved in controlling the size of their patterning domain.

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