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Hox genes interact with the Ras and Wnt signaling pathways to specify anteroposterior body patterns in C. elegans

Author
Maloof, Julin N.

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Hox Genes Interact with the Ras and Wnt Signaling Pathways to Specify Anteroposterior Body Patterns in *C. elegans*

by

Julin N. Maloof

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Preface

There are many people that have contributed to this work and my success in graduate school. Cynthia Kenyon provided endless support, encouragement, and enthusiasm for my work, and helped me place my findings within the larger world of biological research. While providing guidance, Cynthia trusted me to explore my own ideas, thereby allowing me to have much fun, and to grow and develop confidence as a scientist. I particularly appreciate the creative thinking that she brought to bear on my project, and her uncanny insight into scientific mysteries.

I have worked with many wonderful people during my graduate tenure. Bella Albinder made lab much more pleasant, not only by ensuring that the lab was well stocked with supplies and reagents, but also with her warm smile, hearty laugh, and teasing. Kathleen Rañeses, bent over backwards to help me with all kinds of administrative things, and has been a good friend over the years; I will miss morning chats in her office. Raffi Aroian helped me understand *C. elegans* vulval biology, taught me about antibody purification, gave me the idea for the fixation procedure which allowed me to see LIN-39 staining, and most importantly, started the Kenyon Lab wine tasting. Thanks to Lisa Wrischnick for always saying whatever was on her mind. Gregg Jongeward contributed many creative ideas during our post group meeting chats. I am thankful to Ilan Zipkin for his enthusiasm about my work and for bringing his

incredible knowledge of the biological literature to his creative thoughts on my project. Steve Salser is an awe-inspiring person to have worked with. He is an incredibly fast, creative, and broad thinker about biological problems. I particularly appreciate the fact that he always believed that I had something interesting to say, even if I wasn't so sure. Steve also is responsible for developing MAB-5 antibodies, as well as for most of our understanding about the importance of controlling *mab-5* expression. These two contributions made much of my work possible. I would like to thank Bruce Wang for cloning the *C. elegans* Hox cluster, without which none of this would have been possible. More importantly I value Bruce for his close friendship over the years and for showing me that I could achieve goals that I never would have dreamed possible.

I had the opportunity to share my lab bay with three wonderful people. Jeanne Harris is an excellent geneticist, and her love of genetics shows in all of her work. I particularly value Lee Honigberg for his critical thinking, his always asking me what I was going to learn and why I was doing any given experiment. Lee made a number of technical suggestions that led to breakthroughs during protocol development. Craig Hunter was like a second adviser to me,

providing excellent advice, ideas, and forcing me to think hard about my project and about science. Craig also was a great counselor in times of scientific and personal crisis.

I have had wonderful times and received much support from many friends while at U.C.S.F. In particular I would like to thank: Diane Colby for many great river trips and interesting conversations; Jon Tupy for being a wild-and-crazy guy, and for getting me to buy a mountain bike; Deda Gillespie for being a great friend throughout graduate school and for great trips exploring the snowy Sierra; Erik Ullian for his friendship and support and for our almost daily trips to the gym; Jeff Brock for being one of the best friends that I can imagine; and Mark Maloof for being like a brother to me.

Jon Alexander deserves kudos for his heroic reading and editing of my thesis, done with a super-human turnaround time and a smile on his face.

I would especially like to thank Stacey Harmer for being wonderful in every possible way, for a tremendous amount of support, and for making life so fun.

Lastly, my parents. In addition to bringing me into this world, they have had unwavering faith and confidence in me. Throughout

my life, their support have been fundamental in helping me to obtain my goals and dreams.

Abstract

Hox Genes Interact with the Ras and Wnt Signaling Pathways to Specify Anteroposterior Body Patterns in C. elegans

Julin N. Maloof

The diversification of structures along the anteroposterior (A/P) body axis in metazoans depends on region–specific expression of conserved Hox genes. Misexpression of these genes causes fascinating transformations of one body region into another. Limiting expression of the Hox genes to their correct body regions, and controlling fine–scale expression within those regions are both critical for wild–type development. In spite of extensive research in *Drosophila*, as well as research in *C. elegans* and vertebrates, there is still a great deal to be learned about controlling Hox gene expression. I have taken two approaches to investigating mechanisms that control Hox gene expression in *C. elegans*.

First, I screened for mutations affecting the expression of a Hox gene reporter construct One mutation, in a new gene that we named *polyray–1(pry–1)*, caused ectopic expression of all the three Hox genes examined: *lin–39*,*mab–5*, and *egl–5*, showing that wild–type *pry–1* functions to restrict expression of these genes to their local domains. Mutations in the ß–catenin/*armadillo* related gene *bar–1* can suppress mutations in *pry–1* by

preventing ectopic Hox expression. Further studies revealed that these genes function in a conserved Wnt signaling cascade to control both general and fine–scale expression of Hox genes in *C. elegans.*

I also developed antibodies against the Hox gene *lin–39* to better understand its expression and function during development. I found that *lin–39* is upregulated during induction of the *C. elegans* vulva. Vulval development is induced by activation of a conserved Ras signaling pathway; I found that this same pathway is required to upregulate *lin–39* expression. Further investigation revealed that both *lin–39* expression and activation of the Ras pathway are needed for vulval induction to occur, and suggested that *lin–39* functions to specify that vulval fates are adopted as a result of Ras activation. These results identify a new mechanism for controlling Hox gene expression, identify Hox genes as a downstream target for the Ras pathway, and suggest a mechanism for specifying the outcome of Ras signaling.

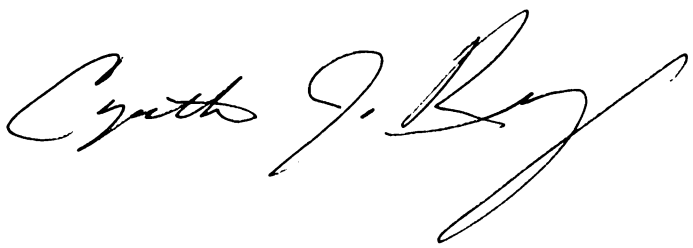

Table of Contents

List of Tables

List of Figures

Chapter One: Anteroposterior body patterns are specified by conserved Hox genes

Understanding how different structures are specified to form in precise locations along the anterior-posterior (A/P) axis of developing animals is a fundamental and fascinating question for developmental biologists. Why do arms, legs, wings, or ribs develop where they do? How are seemingly identical precursor cells assigned different fates? What molecular cues allow cells to detect their position along the A/P axis and develop appropriately? Do cells sense what fates their neighbors have adopted and adjust their fates accordingly? This work describes investigations into mechanisms controlling A/P patterning in the nematode *Caenorhabditis elegans*, undertaken in an effort to better understand the development of this species and to answer some of the questions outlined above.

C. elegans is an excellent organism in which to study A/P patterning. This worm has a short generation time, superb genetics, and is transparent, facilitating observation of cellular differentiation (Brenner, 1974; Wood, 1988). In spite of its apparently simple body plan, there is a surprising amount of differentiation along the worm's A/P axis. In *C. elegans*, as in most metazoans, many A/P decisions are controlled by homeotic selector or Hox genes, which function to provide regional identity to cells along the A/P axis (reviewed by Kenyon et al., 1998; Salser and Kenyon, 1994). Other types of

cell-fate decisions are controlled by signals sent between cells that activate specific signal transduction cascades, leading to changes in gene expression and ultimately cellular differentiation. This work examines how two of the most conserved signaling mechanisms known, the Ras and Wnt systems, interact with the *C. elegans* Hox genes to control A/P patterning and achieve wild-type development.

First described in *Drosophila*, the seemingly magical Hox genes are able to transform the A/P identity of entire segments when mutated. For example, flies with four wings instead of two can be created by mutations that transform halteres into wings; other mutations transform the wings into halteres, thereby producing flies with no wings at all; while still others cause antennae to develop into feet or vice versa (see Lawrence, 1992; McGinnis, 1994) for a historical discussion and review of homeotic genes in *Drosophila*). Elegant genetic studies conducted independently by Lewis and Kaufman demonstrated that two clusters of homeotic genes exist in *Drosophila*, that genes within those clusters worked both individually and in combination to specify segmental fates along the A/P axis, and that the order of those genes along the chromosome was collinear with the regions that they patterned (Kaufman et al., 1980; Lewis, 1978; reviewed by Denell, 1994; Lawrence, 1992).

How might Hox genes function to effect such large developmental changes? When the homeotic complexes were cloned a great deal of excitement was generated because it was found that each cluster contained a

number of genes with a conserved coding sequence (the homeobox), that this sequence was highly conserved in both invertebrates and vertebrates, and that the sequence showed some homology with the yeast mating type loci and bacterial DNA binding proteins (Laughon and Scott, 1984; McGinnis et al., 1984; McGinnis et al., 1984; Scott and Weiner, 1984; Shepherd et al., 1984). Proteins containing a homeodomain were subsequently found to bind DNA, and the Hox genes were shown to act as transcription factors, presumably conferring regional identity by regulating the transcription of downstream target genes that execute appropriate developmental programs (reviewed by Biggin and McGinnis, 1997; Gehring et al., 1994).

One of the most amazing aspects of Hox genes is the degree to which they have been conserved: practically all metazoans examined have a cluster (or clusters in the case of vertebrates) of Hox genes orthologous to those in *Drosophila* (reviewed by Kenyon, 1994; Sharkey et al., 1997). In addition to the conservation of sequence and colinearity, function has been conserved as well: Hox gene mutations in nematodes and mice cause homeotic defects analogous to those originally described in *Drosophila* (Fig. 1.1; see Kenyon et al., 1998; Krumlauf, 1994; Maconochie et al., 1997 for reviews) An even more amazing demonstration of functional conservation was provided by the findings that *C. elegans* and *Drosophila* Hox mutations can be complemented by the orthologous wild-type genes from *Drosophila* and chick, respectively (Hunter and Kenyon, 1995; Lutz et al., 1996).

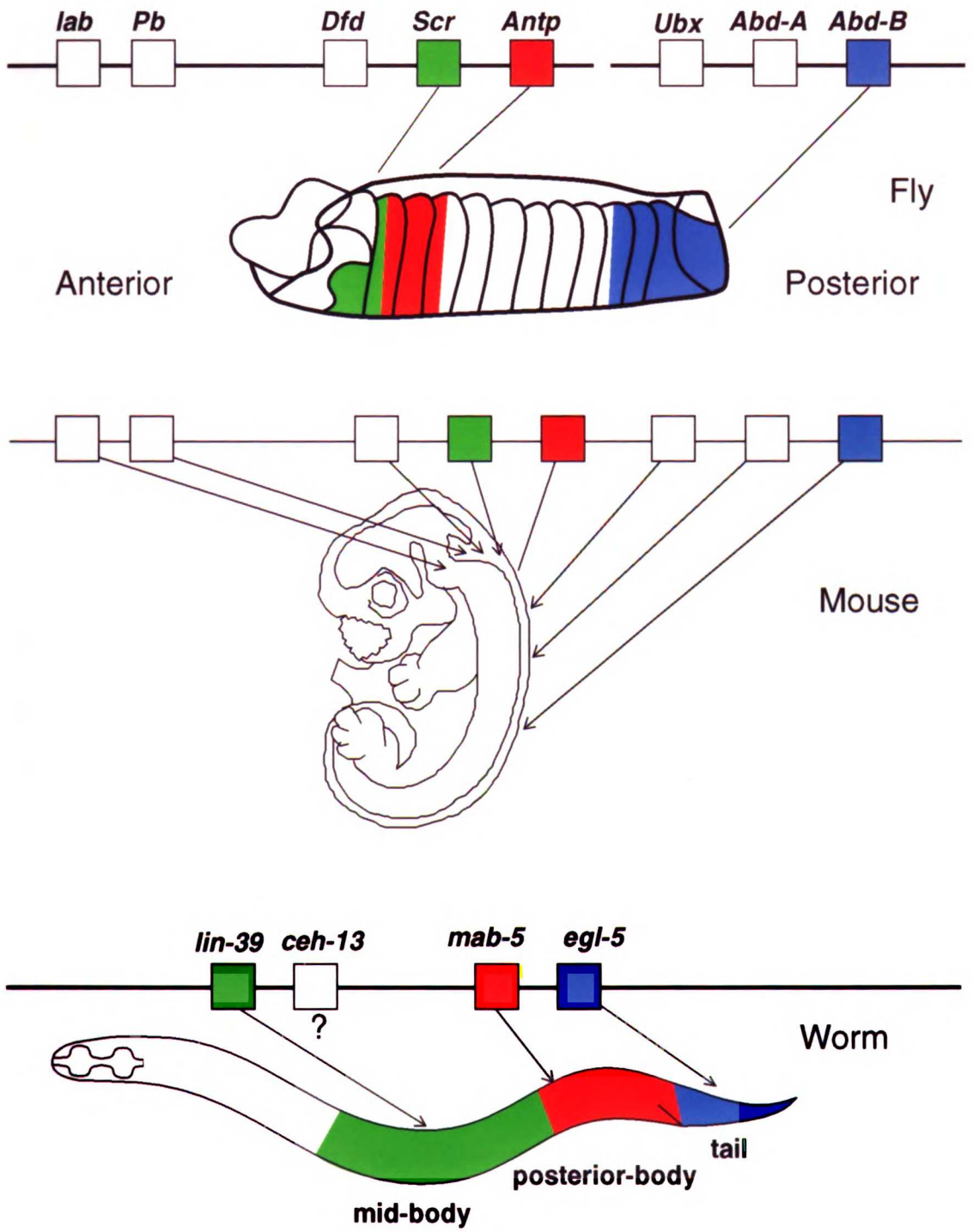

Figure 1.1 Universally conserved Hox genes pattern diverse metazoans.

Conserved clusters of Hox genes pattern the fly (top), mouse (middle), and worm (bottom). Orthologs of genes that are known to be important for A/P patterning in the worm are color coded to match one another.

Hox genes are expressed in broad stripes along the A/P axis that correspond to their domains of function: the genes that pattern the anterior are expressed in the anterior, whereas those that pattern the posterior are expressed in the posterior. Genetic manipulations which either expand or reduce the normal expression domains cause homeotic transformations of one body region to another, not only in flies, but in *C. elegans* and vertebrates as well, demonstrating that these broad patterns of expression are important for correct A/P patterning. Initially it was thought these genes acted on segments as a whole to select the correct identity, and that therefore it was only important that they be either off or on in a given body region (Garcia, 1975; Lawrence and Morata, 1994). While there is some truth to this view, it has since been realized that the patterns of Hox gene expression are spatially intricate and temporally dynamic, and that these complex expression patterns are essential for achieving correct development. For example, *Drosophila* parasegment 5 is given its unique identity by fine-scale control of *Ubx* expression; ubiquitous *Ubx* expression gives a parasegment 6 identity (Castelli-Gair and Akam, 1995). Similarly, the *C. elegans* Hox gene *mab-5* is switched on and off multiple times in the V5 cell lineage, and each change in expression is necessary for a particular patterning decision (Salser and Kenyon, 1996). The same may be true in vertebrates: during limb outgrowth the Hox genes are expressed in a highly dynamic pattern, and changes in expression correlate with the production of different limb segments,

suggesting that the changing patterns may be important for specifying these different segments (Nelson et al., 1996). Thus, having the Hox genes expressed in both their correct broad and intricate patterns is crucial for correct A/P specification to occur. Understanding the mechanisms that control Hox gene expression is therefore critical to answering the broader questions of how A/P patterns are specified. This question is also interesting from an evolutionary point of view: *Drosophila,* mice, and *C. elegans* all appear to have fundamentally different methods of embryonic development, and yet in all cases the Hox genes come to be expressed in similar patterns. Will the mechanisms that control Hox gene expression be conserved in all of these species in spite of their different organization? If so, how have these mechanisms adapted to the different strategies of embryonic development? Alternatively, how have different control mechanisms evolved to give the same expression patterns?

Most of what is known about control of Hox gene expression comes from work on *Drosophila,* and most of this work has focused on understanding how the initial patterns of expression are set up in the early embryo. In *Drosophila* maternally encoded factors such as *bicoid* lead to gradients of zygotic gap gene expression, which in turn initiate striped expression of the Hox genes. The initial Hox patterns are refined by pair-rule genes such as *ftz* and *eve* to bring the boundaries of Hox expression into precise alignment with the parasegmental borders (early Hox expression

controls are reviewed in Lawrence, 1992). After these initial expression patterns are achieved they are maintained through the combined actions of the *Polycomb*-group and *trithorax*-group genes, which function to maintain transcriptional repression and activation, respectively. Because of their homology to chromatin binding proteins and the yeast Sin/Swi proteins, these genes are thought to act, at least in part, by maintaining chromatin in ether active or inactive states reviewed by Pirrotta, 1997; Tamkun, 1995). Later, after the initial ectodermal Hox patterns have been set up, *Wingless* signaling is important for patterning Hox expression in the midgut: expression of *Wingless* in the visceral mesoderm maintains *Ubx* expression as part of an autoregulatory loop and also controls *labial* expression in the underlying endodermal cells (Hoppler and Bienz, 1995; Riese et al., 1997; Thuringer et al., 1993). Beyond this, little is known about late induction or modification of Hox expression patterns in *Drosophila*.

In vertebrates the regulation of Hox genes may be even more complex than in invertebrates (e.g. Nelson et al., 1996; see also review by Maconochie et al., 1996). Two secreted signaling proteins are known to be important for inducing Hox expression: Sonic hedgehog and FGF. *Sonic hedgehog (Shh)* expression both in the limb zone of polarizing activity (ZPA) and in the developing gut has been shown to induce and pattern Hox expression in nearby cells (Riddle et al., 1993; Roberts et al., 1995). Regulation of Hox responsiveness to Shh leads to dynamic temporal and spatial patterns of

expression, although the mechanism for this modulation is unknown (Nelson et al., 1996). FGFs are important for expression of *Hoxd-13* in the limb bud, and enable limb cells to respond to Shh (Vogel et al., 1995). Although less well characterized, it is likely that retinoids also play a role in patterning Hox expression, since exogenous retinoic acid can cause activation of different Hox genes in a concentration dependent manner, and because retinoic acid response elements have been found in the promoter regions of several murine Hox genes (reviewed by Marshall et al., 1996). It is not clear, however, where endogenous retinoids are produced, nor how important they are during wild-type development. Interestingly, genes with sequence homology to the *Drosophila Polycomb*-group and *trithorax*-group genes are important for controlling Hox expression in vertebrates as well (reviewed by Gould, 1997). Although both the invertebrate and vertebrate studies provide insights into the various mechanisms that regulate Hox gene expression, they only provide a few examples of what is likely to be a very complex and diverse set of controls. Clearly a great deal remains to be learned about how Hox gene expression patterns are initiated in different systems, about how those patterns are modified, and about how the Hox genes function to correctly specify A/P pattern.

As mentioned above, studies in *Drosophila* indicate that Hox gene expression can be controlled by members of the Wnt family of extracellular signaling molecules. *Wnt* genes encode secreted glycoproteins that trigger a

well-conserved signal transduction cascade in nearby cells, leading to changes in cell specification and polarity (Fig. 1.2; reviewed by Cadigan and Nusse, 1997). Activation of the Wnt signaling cascade culminates in the translocation of the Beta-catenin/Armadillo transcriptional coactivator into the nucleus, where it forms a complex with Lef-1/TCF that activates transcription of target genes (reviewed by Miller and Moon, 1996; Nusse, 1997).

Wnt proteins are involved in a remarkable array of inductive signaling events in organisms ranging from *C. elegans* to mammals (reviewed by Cadigan and Nusse, 1997; Moon et al., 1997). They are, for example, required for segment polarity, midgut induction, and dorsoventral patterning of appendages in *Drosophila*, and dorsoventral patterning of limbs, kidney induction, and CNS patterning in mice. In *C. elegans*, Wnt signaling is required during early embryogenesis for correct blastomere cytoskeletal polarity and for endoderm induction (Rocheleau et al., 1997; Thorpe et al., 1997). Later, during larval development, Wnt signals provide correct polarity to a number of asymmetric cell divisions (Herman et al., 1995; Sawa et al., 1996). Although the Wnt molecule *Wingless* controls Hox gene expression in the midgut of *Drosophila*, it is not known if Wnts are widely used as a Hox control mechanism in metazoan development.

In contrast to Wnt signaling, there is little evidence of transcriptional control of Hox genes by activation of the Ras pathway (although transcription

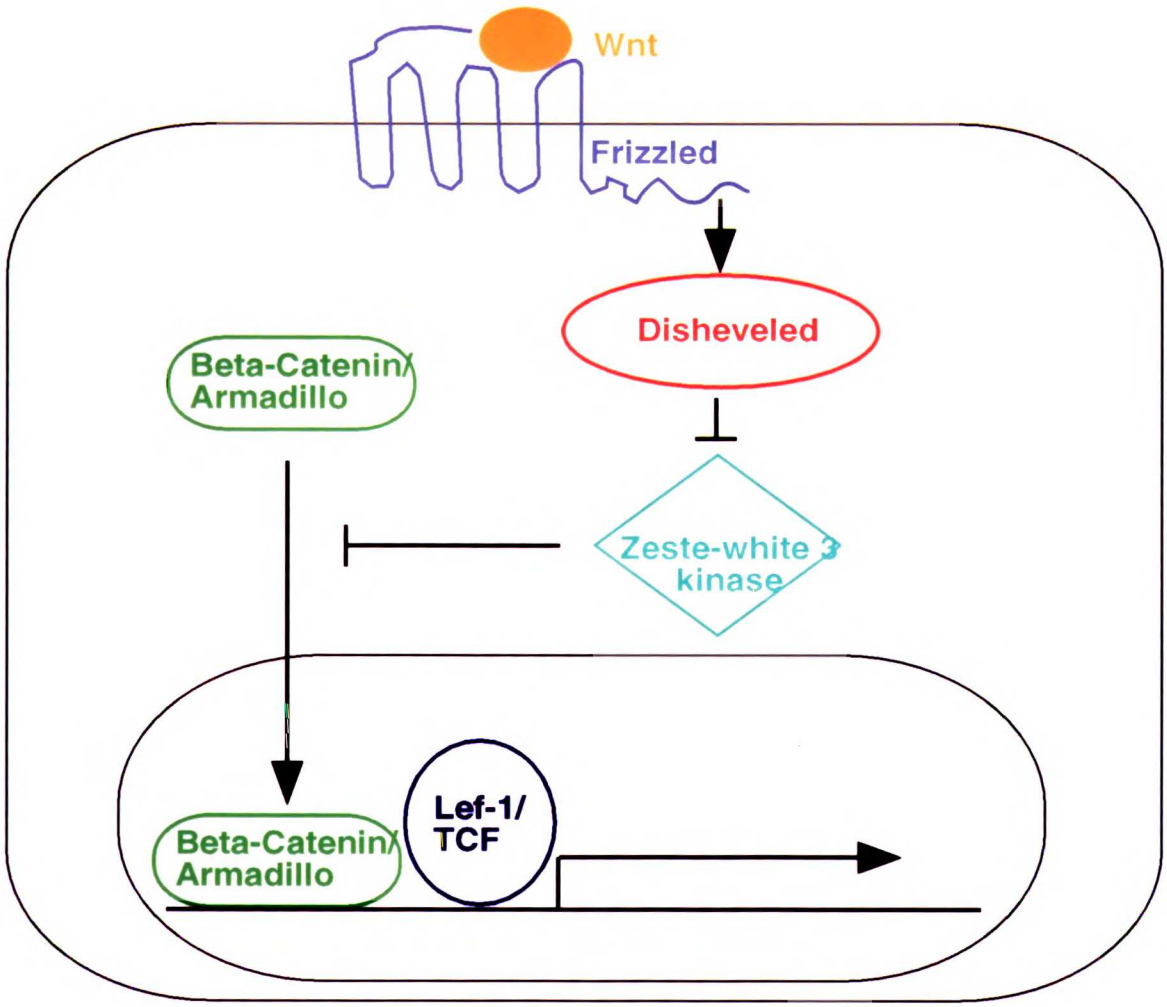

Figure 1.2 The conserved Wnt signaling cascade.
Binding of Wnt to its receptor, the seven transmebrane protein Frizzled, leads to activation (directly or indirectly) of the phosphoprotein Disheveled. Through unknown mechanisms, activated Disheveled is thought to inhibit activity of the Zeste-white 3 kinase (it is also possible that Disheveled and Zeste-white 3 function parallel to one another). In unstimulated cells, Zeste-white 3 acts to promote degradation of Beta-Catenin/Armadillo. Therefore, inhibition of Zeste-white 3 kinase upon Wnt activation causes an accumulation of Beta-Catenin/Armadillo, leading to its translocation into the nucleus, where it acts with Lef-1/TCF transcription factors to activate transcription.

independent modulation of Hox function by Ras in *Drosophila* has recently been reported (Boube et al., 1997)). Ras, a small GTPase, is the central member of a highly conserved signaling cascade (Fig. 1.3; reviewed by Egan and Weinberg, 1993; Kayne and Sternberg, 1995; Wassarman et al., 1995). Activation of this pathway is important for specifying a number of cell fates, including terminal fates and photoreceptor identity in *Drosophila*, and vulval fates in *C.elegans* (Kayne and Sternberg, 1995; Wassarman et al., 1995). Unregulated activation of the Ras pathway has also been implicated in numerous cancers. Although there is a "cassette" of highly conserved proteins involved in Ras signal transduction, few genes functioning downstream of the cassette have been identified. Furthermore, activation of this cassette leads to a very diverse set of responses in different tissues, yet the factors which specify these varied outcomes of Ras signaling in particular cell types are not well understood.

This work addresses the role of extracellular signals in the control of Hox gene expression and the specification of A/P fates in three *C. elegans* cell types: the migratory Q neuroblasts, the lateral epidermal V cells, and the ventral epidermal Pn.p cells. *C. elegans* has a cluster of four Hox genes: *lin-39*, a *Sex combs reduced* homolog; *ceh-13* a *labial* homolog; *mab-5*, an *Antennapedia* homolog; and *egl-5*, an *AbdominalB* homolog (Kenyon and Wang, 1991). At least three of these genes, like their counterparts in other organisms, are expressed in blocks of cells along the A/P axis collinear with

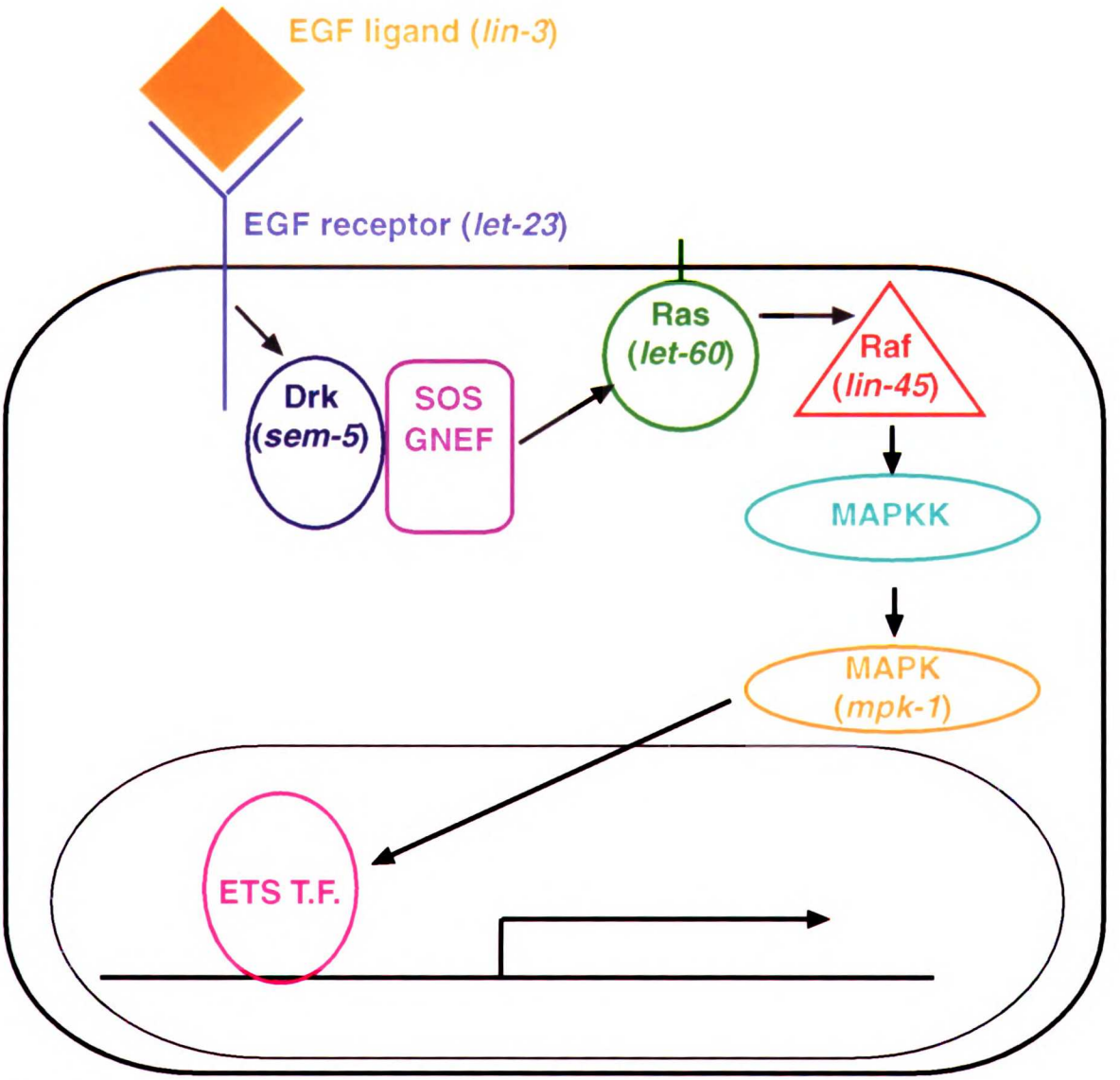

Figure 1.3 The conserved Ras pathway.
Genes encoding *C. elegans* homologs of the proteins shown are enclosed in parentheses. The signaling cascade is activated when an EGF-type ligand binds to an EGF-receptor tyrosine kinase, leading to receptor autophosphorylation. The adaptor protein GRB2/Drk (Downstream of receptor kinase) binds both to phosphorylated receptor and to a guanine nucleotide exchange factor (GNEF; Sos in Drosopihla), causing activation of Ras by the exchange of GDP for GTP (Ras is returned to its inactive state when its intrinsic GTPase activity is stimulated by a GTPase-activating protein or Gap, not shown). Activated Ras recruits the serine/threonine kinase Raf (also known as MAPKKK) to the plasma membrane, where it is activated in a process which likely requires the 14-3-3 protein (not shown; Li et al., 1997). Raf, once activated, phosphorylates MAPKK, which, in turn, phosphorylates and activates MAPK. MAPK leads to changes in transcription by phosphorylating ETS domain transcription factors.

their order on the chromosome, and are required where they are expressed to provide regional identity: *lin-39* patterns the mid-body, *mab-5* the posterior, and *egl-5* the tail (Fig 1.4; (Chisholm, 1991; Clark et al., 1993; Costa et al., 1988; Kenyon, 1986).

The specific fates adopted by the Q and V cells depend critically on the particular Hox gene expressed in the cell. The two Q cells are born on opposite sides of the animal but in identical A/P positions. However, a short migration separates them soon after hatching: QL, the cell on the left, migrates towards the posterior and then switches on *mab-5*, whereas QR, the cell on the right, migrates towards the anterior without expressing *mab-5*. After their migration, these cells divide and their descendants continue to migrate: those of QL continue into the posterior, whereas those of QR continue towards the anterior. Correct expression of *mab-5* is critical for these migrations: in the absence of *mab-5* the descendants of QL migrate to the anterior like the wild-type QR descendants, whereas if *mab-5* is ectopically provided to QR, its descendants migrate into the posterior as if they had adopted the normal QL descendant fate (Salser and Kenyon, 1992). Adjacent to the Q cells, there are two rows of V cells, one on each side of the worm; these rows stretch from nearly the anterior to posterior ends of the worm. The V cells in the anterior (V1-V4) differentiate to make a ridged cuticular structure known as alae, while in males, the more posterior cells V5 and V6 differentiate to make neuronal sensory structures called rays (in

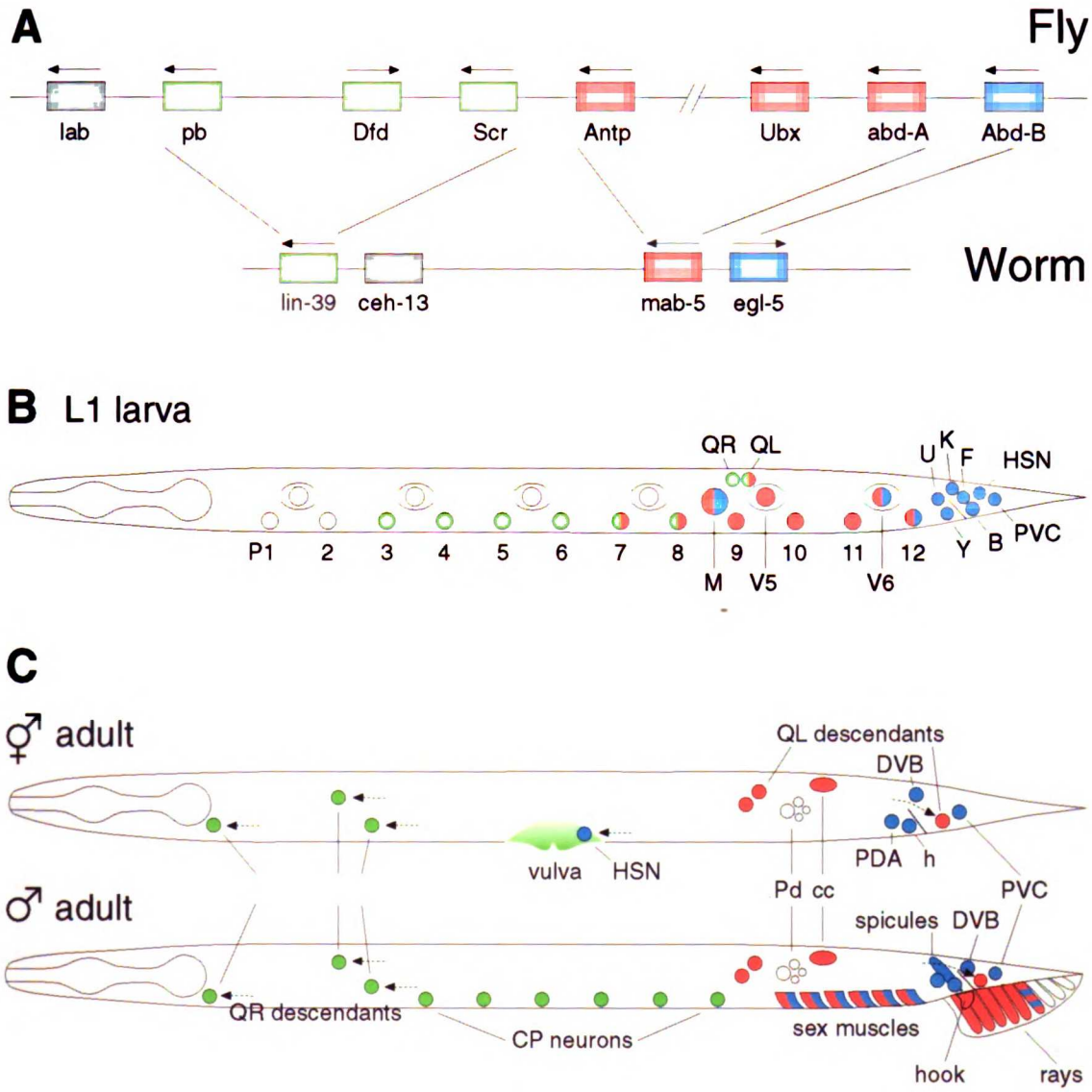

Figure 1.4 Organization and patterning function of the *C. elegans* Hox cluster. (A) Probable evolutionary relationships between *C. elegans* and *Drosophila* Hox genes.
(B) Diagram of newly hatched L1 larva (male and hermaphrodite combined) showing cells that require the function of *lin-39* (green), *mab-5* (red), and *egl-5* (blue) to develop correctly. These cells give rise to the structures shown in the adult in (C)
(C) Diagram of an adult worm, again color coded to represent the Hox gene(s) required for their development. Not all structures and cell types specified by the Hox genes are shown. Dashed lines indicate cell migrations. cc, coelomocyte; PVC, PDA, DVB, and the Q descendants, neurons; CP, serotonergic Pn.aapp neurons; h, hypodermal cell. Adapted from Wang et al. (1993).

hermaphrodites all V cells make alae). Here again *mab-5* acts as a genetic switch between different fates: *mab-5* is expressed in the cells that will make rays, where it is both necessary and sufficient for ray production (Salser and Kenyon, 1996).

What controls the patterns of Hox gene expression in *C. elegans*? Although a coherent picture has yet to emerge, some of the genes affecting Hox expression in *C. elegans* show homology to *Drosophila* Hox regulators, suggesting that some aspects of Hox control have been conserved. For example, *vab-7*, an *eve* homolog, regulates expression of the posterior Hox gene *egl-5* in the tail (Ahringer, 1996), and*pal-1*, a *caudal* homolog, is required for the expression of *mab-5* in the posterior cell V6 (Salser and Kenyon, 1996); these results suggest conservation of posterior patterning mechanisms. Mutations in *lin-22*, a *hairy/enhancer of split* homolog, have the interesting effect of causing the V5 lineage to be reiterated in the anterior V cells V1-V4. In addition, V1-V4 express the Hox gene *mab-5* in a pattern resmbling wild-type *mab-5* expression in V5 (Wrischnik and Kenyon, 1997), suggesting that *lin-22*, like *hairy*, functions as a transcriptional repressor (although it is possible that the ectopic *mab-5* expression is a relatively indirect consequence of the V1-V4 to V5 transformation). Not all homologies are revealing: *lin-17*, a *frizzled* type receptor, is required for proper expression of *mab-5* in QL (Harris et al., 1996; Sawa et al., 1996), but a role for *frizzled* in Hox expression in other species has not been reported. In spite of some similarities, the

initiation of Hox expression may be controlled by very different mechanisms in the two species. In *Drosophila*, Hox expression is initiated by A/P gradients of diffusible morphogens; in contrast, (Cowing and Kenyon, 1996) found that in *C. elegans*, initiation of *mab-5* expression in V6 and the migratory cell M does not depend on A/P position, suggesting that in *C. elegans*, initiation of Hox expression may not depend on localized positional cues. Clearly there are many questions remaining about the control of Hox gene expression in *C. elegans*, and the genes identified so far probably represent only a small fraction of the inputs controlling Hox gene expression. Chapter 2 details a screen for mutations affecting the expression of a *mab-5* reporter construct, performed in an effort to gain a better understanding of Hox control in *C. elegans*. That screen enabled identification of a gene, *polyray-1 (pry-1)*, that plays an important general role in repressing Hox genes throughout *C. elegans*. Further studies have revealed that *pry-1* functions as part of a Wnt signaling cascade, and that activation of this Wnt cascade is required for switching on *mab-5* in QL.

The row of Pn.p cells, like the two rows of V cells, stretches almost the entire length of the animal. These cells differentiate depending on both the sex of the animal and their A/P position. In both sexes there are two different specifications that occur: first some of the Pn.p cells fuse into a multinucleate syncytium, and then later a subset of the unfused cells undergo multiple rounds of division and morphological changes leading to the production of a

sex-specific organ. In hermaphrodites the anterior and posterior cells fuse, whereas the central cells remain unfused and later differentiate to make the vulva. In males the fusion pattern is different: the most posterior cells, along with a smaller subset of the central cells remain unfused. Eventually the most posterior of these unfused cells divide to generate the Pre-Anal Ganglion and hook (collectively referred to as the PAG), a set of neurons, support cells, and structures important for male mating.

The first step in this developmental process, the decision to either fuse or remain unfused, is controlled by Hox genes: in hermaphrodites those cells that express *lin-39* remain unfused and those that do not fuse. In males, expression of either *lin-39* or *mab-5* is sufficient to prevent fusion, but cells expressing both or neither Hox genes fuse (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993). The second step, organ development, is controlled in part by Ras signaling. Activation of the Ras signaling cascade in hermaphrodite Pn.p cells is necessary for vulval fates to be adopted (see Eisenmann and Kim, 1994; Kenyon, 1995 for reviews), and the Ras pathway also plays an important role in patterning the PAG fates in males (Paul Sternberg, personal communication).

Interestingly, although the development of the vulva and PAG takes place within the domains of *lin-39* and *mab-5* respectively, it was not known if the Hox genes were involved in the development of either of these organs. Chapter 3 examines the role of *lin-39* in development of the vulva, and to a

lesser extent the role of *mab-5* in the PAG. These studies led to a number of interesting findings. First, Ras signaling can control *lin-39* expression, and *lin-39* is required for Pn.p response to Ras activation. This demonstrates a new mechanism for Hox control and identifies a new gene functioning downstream of the Ras signaling cassette. Second, Hox genes are able to provide specificity to Ras signaling, a finding which provides insight into the problem of how Ras can specify different fates in different locations in the animal.

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Chapter Two: the *pry-1* and *bar-1* genes function in a conserved Wnt signaling pathway to control both general and fine-scale Hox gene expression

SUMMARY

The patterning of cell fates along the anteroposterior (A/P) body axis is achieved in a large part by the actions of conserved clusters of Hox genes. Limiting expression of these genes to their normally localized regional domains, as well as controlling their precise patterns of expression within those domains is critically important for specifying wild-type development. We describe a *C. elegans* gene *pry-1* which is required to limit expression of the Hox genes *lin-39, mab-5,* and *egl-5* to their correct local domains; when *pry-1* is mutated, all three genes become ectopically expressed. This misexpression phenotype can be blocked by mutations in a second gene, *bar-1* which encodes a ß-catenin/armadillo related protein. We go on to show that these two genes function in a conserved Wnt signaling pathway which is required to activate expression of *mab-5* in the migratory neuroblast QL. Our results show that components of the Wnt signaling system play a major role in both the general and fine-scale control of Hox gene expression in *C. elegans*

INTRODUCTION

Conserved Hox genes are required to specify regional identity along the

metazoan anterior/posterior (A/P) axis. Three of the four *C. elegans* Hox genes, like their *Drosophila* and vertebrate homologs, have been shown to be expressed in broad stripes along the A/P axis, and to function where they are expressed to specify regional identity: *lin–39*, a *Sexcombs reduced* homolog, patterns the mid–body, *mab–5*, an *Antennapedia* homolog, the posterior, and *egl–5*, an *AbdominalB* homolog, the tail (Chisholm, 1991; Clark et al., 1993; Wang et al., 1993).

Limiting the expression of the Hox genes to their appropriate regions is essential for correct patterning. For example, expression of *lin–39* in the mid–body of hermaphrodites directs one particular ectodermal cell type, the Pn.p cells, to make a vulva in response to inductive signals; if the posterior gene *mab–5* is expressed in the mid–body in place of *lin–39*, then mid–body Pn.p cells adopt PAG fates and make a mating structure normally produced by Pn.p cells in the posterior of males (Maloof and Kenyon, 1998). Restriction of *mab–5* to the posterior is also important in the V cells, lateral ectodermal cells that stretch in a row along each side of the worm. Normally *mab–5* is only expressed in the descendants of the posterior–most V cells; in males this expression specifies the production of sensory structures known as rays, instead of the ridged cuticular structures, known as alae, made by anterior cells. Ectopic expression of *mab–5* in the anterior causes the anterior V cells to adopt the posterior fate, making rays instead of alae (Salser and Kenyon, 1996).

How are Hox gene expression patterns restricted to the appropriate A/P region? This is best understood in *Drosophila*, where gradients of maternal factors cause regional expression of the gap genes, which, in turn, activate Hox expression in broad A/P stripes. The initial patterns are refined by the pair–rule genes (reviewed in Lawrence, 1992; Lawrence and Morata, 1994), and then maintained largely by the action of the *Polycomb*–group and *trithorax*–group genes (reviewed by Pirrotta, 1997; Tamkun, 1995).

Early embryonic development in *C. elegans* and *Drosophila* appear quite different: *C. elegans* embryogenesis is characterized by asymmetric blastomere cleavage and lineal segregation of fates whereas embryogenesis in *Drosophila* begins with synchronous rounds of nuclear division creating a syncytium of identical nuclei that are patterned by diffusible morphogen gradients. It is likely that the mechanisms that control patterned Hox expression have diverged as well (Kenyon, 1994). For example, (Cowing and Kenyon, 1996) found that in certain cells *mab–5* expression can be initiated correctly, independent of A/P position (also see Kenyon et al., 1998). Nevertheless, some of the genes that control Hox expression in *C. elegans* are homologs of important patterning genes in *Drosophila*: *vab–7*, an *eve* homolog, regulates expression of *egl–5* in the tail (Ahringer, 1996), and *pal–1*, a *caudal* homolog, is required for the expression of *mab–5* in the posterior cell V6 (Salser and Kenyon, 1996); these homologies suggest that posterior patterning mechanisms are conserved between the two species. Mutations in

lin–22, a *hairy/enhancer of split* homolog, have the interesting effect of causing the V5 lineage to be reiterated in the anterior cells V1–V4. The Hox gene *mab–5* is expressed ectopically in these cells(Wrischnik and Kenyon, 1997), suggesting that the *hairy* homolog has retained its function as a transcriptional repressor. However, the ectopic *mab-5* expression may be an indirect result of the V1-V4 to V5 transformation, since this transformation does not require *mab-5*. *lin–22* may act similarly to the *Drosophila Polycomb*–group genes to maintain patterns of Hox expression; alternatively, the ectopic *mab–5* expression may be a relatively indirect consequence of the transformation to the V5 fate. Mutations in the several *C. elegans Polycomb* type homologs that have been found cause maternal effect sterility (Garvin et al., 1998), however these have only low penetrance homeotic defects (J. Maloof and C. Kenyon, unpublished), so it is not yet clear how important they are for Hox gene regulation.

In some cases Hox genes must be expressed in precise spatial and temporal patterns to achieve correct development. For example, *Drosophila Ubx* is expressed in an intricate pattern in parasegment 5, and this expression is crucial for giving parasegment 5 its unique identity (Castelli-Gair and Akam, 1995). Hox expression patterns are very dynamic in vertebrates, and it is likely that in at least some cases this dynamic expression is required for proper development (e. g. Nelson et al., 1996). Likewise, precise spatial and temporal control of *mab–5* expression is crucial for achieving wild–type

development of at least two cell types, the V cells and the migratory Q neuroblasts. In the V5 lineage, *mab–5* is switched ON and OFF multiple times, and each change in expression specifies a different patterning decision (Salser and Kenyon, 1996). The two Q cells are neuroblasts born in identical A/P positions but on opposite sides of the worm: QL on the left and QR on the right. In wild–type worms, after a short posterior migration, QL switches on *mab–5*, and, as a result, the descendants of QL remain in the posterior (Salser and Kenyon, 1992). In contrast, QR migrates towards the anterior, *mab–5* remains off in QR and its descendants, and as a result the descendants of QR continue to migrate towards the anterior. *mab–5* acts as a switch to control the direction of Q–descendant migration: in the absence of *mab–5* activity, the descendants of both Q cells migrate towards the anterior, and conversely, ectopic expression of *mab–5* in both cells causes the descendants of both cells to stay in the posterior.

A number of genes have been described that, without affecting the initial migration of the Q cells, are required for *mab–5* to be switched on in QL (Harris et al., 1996). Recently it has been found that one of these, *egl–20*, encodes a Wnt type signaling molecule (J. Whangbo and C. Kenyon, in preparation), and that two others, *mig–1* and *lin–17*, encode homologs of *Drosophila frizzled* and likely act as Wnt receptors (S. Clark, personal communication; (Sawa et al., 1996). Additionally, *mig–5* is required for correct migration of the QL descendants, and encodes a homolog of

disheveled, which is thought to act downstream of *frizzled* in Wnt signal transduction (Guo, 1995). Together these results suggest that Wnt signaling is required for switching on *mab–5* in QL.

The Wnt family of extracellular signaling molecules provides positional information and establishes polarity in diverse tissues in many different organisms (reviewed by Cadigan and Nusse, 1997). Typically, Wnt signals activate a conserved signal transduction cascade that leads to translocation of ß–catenin/armadillo into the nucleus(Peifer et al., 1994). Once in the nucleus ß–catenin/armadillo acts in concert with TCF/LEF–1 related transcription factors to activate expression of downstream genes such as *engrailed*, and the Hox gene *Ubx* (van de Wetering et al., 1997); reviewed by Miller and Moon, 1996; Nusse, 1997). In the absence of Wnt signals, most ß–catenin/armadillo is found complexed with cadherins at adherens junctions. This is in part due to the *zeste–white 3* kinase, which, in the absence of Wnt signals, promotes rapid degradation of cytoplasmic ß–catenin/armadillo (Peifer et al., 1994). Activation of the Wnt pathway may cause nuclear translocation of armadillo by inhibiting zeste–white 3 kinase activity (Orsulic and Peifer, 1996; Pai et al., 1997). Wnt signals can also be transduced through a second, less well defined pathway that is important for planar tissue polarity in *Drosophila* (Park et al., 1996; Strutt et al., 1997; Wong and Adler, 1993; also see Cadigan and Nusse, 1997).

Wnt signaling is important for multiple developmental processes in *C.*

elegans, including establishment of embryonic polarity, where a full genetic pathway (with some modifications) has been uncovered (Rocheleau et al., 1997; Thorpe et al., 1997), and determination of epidermal polarity, where Wnt molecules and receptors have been implicated (although in this case the downstream effectors are unknown (Herman et al., 1995; Sawa et al., 1996)). As the genes previously implicated in activation of *mab–5* in QL represent only the upstream part of the Wnt signaling cascade, it is not clear whether downstream signaling takes place via a conserved *armadillo* pathway, or through a different pathway, such as the less well defined pathway required for *Drosophila* cell polarity.

Knowledge of how both broad regional and intricate spatio–temporal patterns of Hox gene expression are achieved is critical for understanding A/P patterning. Here we describe two genes which function in a Wnt pathway to control *mab–5* expression in the V cells and Q cells. One gene, *pry–1* is required to inhibit activation of this pathway; the second, *bar–1*, a *ß–catenin/armadillo* homolog (Eisenmann et al., in preparation), functions to transduce Wnt signals. Interestingly, mutations in *pry–1* cause dramatic, widespread ectopic expression of all Hox genes examined, suggesting that *pry–1* acts as a general repressor of *Hox* expression and that inhibition of Wnt signaling is important for preventing ectopic Hox expression. Furthermore, we have found that *bar–1* is required for activation of *mab–5* in QL, demonstrating that the Wnt pathway is used to control fine–scale Hox gene

expression, and that a conserved Wnt pathway functions to control the direction of Q cell descendant migration.

MATERIALS AND METHODS

General Methods and Strains

Strains were maintained using standard methods (Brenner, 1974; Wood, 1988). Unless otherwise noted, strains were maintained and analyzed at 25° C. The mutant alleles used are either described by this paper, described by Wood (1988), or referenced below.

Mutations used

LGI: *pry–1(mu38)*, *pry–1(nc1)* (S. Takagi, personal communication), *mig–1(e1787)* (Desai et al., 1988), *lin–17(n671)* (Ferguson and Horvitz, 1985), *unc–101(m1)*, *glp–4(bn2)* (Strome et al., 1994), *lev–10(x17)*.

LGII: *mig–14(mu71)* (Harris et al., 1996), *muIs32[mec-7::GFP, lin-15(+)]* (Queelim Ch'ng, Mary Sym, and C.K., unpublished).

LGIII:*mab–5(e2088)*(Hedgecock et al., 1987)

LGIV: *egl–20(n585)*, *him–8(e1489)*.

LGV: *him–5(e1490)*, *muIs3[mab–5–lacZ + pRF4(rol–6d)]* (Cowing, 1992; Salser and Kenyon, 1992), *muIs13 [egl–5–lacZ + pRF4(rol–6d)]* (Wang et al., 1993)

LGX: *bar–1(ga80)* (Eisenmann et al., in preparation); *bar–1(mu63)*, *bar–1(mu349)*, *unc–6(n102)*, *dpy–7(e88)*.

The strain RW7000 (Williams et al., 1992) was used for STS mapping.

Isolation of mutants

pry–1(mu38) was isolated in a screen for mutations affecting *mab–5–LacZ* expression. *mab–5(e2088); muIs3* worms were mutagenized with 25–50mM Ethymethanesulfonate (EMS; Sigma) using standard procedures (Wood, 1988). F2 worms were picked clonally to individual wells of 12–well cluster plates filled with NG agar media. Staged populations of F4s were collected as late L1s and then stained with Xgal, as described below.

pry–1(nc1) was identified in a screen for neuroanatomical defects using EMS as the mutagen (S. Takagi, personal communication).

bar–1(mu63) arose spontaneously in a *pry–1(mu38); muIs3* background. It was noticed because the strain no longer showed the Pry–1 plate phenotype.

bar–1(mu349) was identified by screening a *mec–7–GFP* strain for mutations affecting the migration of the QL and QR descendants (Mary Sym, Queelim Ch'ng, and C.K., unpublished).

Genetic Mapping

pry–1. STS mapping (Williams et al., 1992) was used to map *pry–1(mu38)* to the right arm of chromosome I, and closely linked it to the markers Tcbn2 and hp4: 1/105 homozygous *mu38* F2s picked up both markers and 1/105 picked up just hp4. Additional STS mapping was done by crossing an *unc–101(m1) pry–1(mu38)* double mutant strain to RW7000. 0/5 Pry non–Unc recombinants picked up the markers, whereas 7/11 Unc non–Pry

worms picked up the markers, suggesting that *pry–1* maps to the right of Tcbn2 and hp4. However, this conclusion is based on absence of Tcbn2 and hp4 from 4 Unc non–Pry worms; it is also possible that the marker bands from these worms were missing because of a poor DNA prep. Three–factor mapping placed it between *glp–4* and *lev–10*: from *glp–4(bn2) lev–10(x17)/pry–1(mu38)* heterozygotes, 5/9 Lev non–Glp recombinants segregated Pry progeny.

bar–1. STS mapping (Williams et al., 1992) was used to map *bar-1(mu63)* to the middle of chromosome X, between the markers Stp103 and Stp156. Three–factor mapping placed it between *unc–6* and *dpy–7*: from *unc–6(n102) dpy–7(e88)/bar–1(mu63)* heterozygotes, 4/8 Dpy non–Uncs segregated Bar progeny.

pry–1 recessiveness

mec–7–GFP males were crossed to *pry–1(mu38); him–5(e1490)* or *pry–1(nc1); him–8(e1489)* hermaphrodites. All glowing F1s were found to have the QR descendants in a wild–type position and to have normal alae/ray patterns.

Complementation tests

pry–1. *pry–1(mu38)/+ ; mec–7–GFP/+; him–5(e1490)* males were crossed to *nc1; him–8(e1489)* hermaphrodites. Approximately one half of the glowing progeny had mispositioned QR descendants (posterior of the vulva), and about one half of the glowing male progeny had no alae but instead had

ectopic rays.

bar-1. To show that *mu63* and *mu226* were alleles of *bar-1, him-5(e1490); mu63* or *him-5(e1490); mu226* males were crossed to *unc-30(e191); bar-1(ga80)* hermaphrodites. From each cross, all non-Unc hermaphrodite progeny had mutant QL descendants (anterior of ALM). To show that *mu349* was an allele of *bar-1*, male *mec-7-GFP* worms were crossed to *bar-1(mu226)* hermaphrodites. The resulting *mec-7-GFP/+; bar-1(mu226)/0* males were crossed to *mu349* hermaphrodites. 6/6 glowing hermaphrodite progeny from that cross were found to have QL descendants in a mutant (anterior of ALM) position.

Double mutant construction

pry-1(mu38) doubles with *mig-1(e1787), mig-14(mu71),* and *egl-20(n585)* were all made essentially the same way. *mig-1, mig-14,* or *egl-20* mutant males were crossed to *pry-1* mutant hermaphrodites. Non-Pry F1s were picked. The *mig-1, mig-14,* or *egl-20* mutant alleles were homozygosed first, by picking F2s that had QL descendants in the anterior. Subsequently *pry-1(mu38)* was homozygosed by picking F3s with QR descendants in the posterior. The presence of *mig-1, mig-14,* and *egl-20* in the strains was confirmed by observing the HSN migration defect (Desai et al., 1988; Harris et al., 1996).

The *pry-1(mu38); bar-1(mu349)* double was made by crossing

pry–1(mu38)/+ males to *bar–1(ga80)* hermaphrodites. F2 *pry–1(mu38)* progeny were picked based on their mutant QR descendant positions. Subsequently *bar–1(mu349)* was homozygosed by picking animals with mispositioned QL descendants. A complementation test was done to confirm the presence of *pry–1(mu38).*

Sequencing *bar–1* alleles.

To isolate total RNA, approximately 100µl of mixed stage *bar–1(mu63)* or *bar–1(mu349)* worms were rinsed 3 times in dH_2O and then frozen in liquid N_2. For each tube, 1 ml Trizol reagent (Gibco BRL) was added to the worm pellets and then vortexed hard for 10 minutes. After a 5 minute 25°C. incubation, and a 5 minute spin at full speed in an Eppendorf microfuge, the bottom layer was removed to a fresh tube and then vortexed with 200µl choloroform for 15 seconds. After another spin (as above), the upper, aqueous phase was removed to a fresh tube and mixed with 500µl isopropanol. After ten minutes at room temperature RNA was collected by a 10 minute spin (as above). The pellet was washed with 75% ethanol in DEPC-treated dH_2O, spun again for 5 minutes at 7.5K RPM, allowed to air dry for 20 minutes and resuspended in 100µl DEPC-treated H_2O. First strand synthesis was done using SuperScript II (Gibco BRL) and a dT20 primer. PCR with the Pwo enzyme (Boehringer) was used to amplify the cDNA in three overlapping fragments: A) 830 b.p. from primer SL1

(5'–GGTTTAATTACCCAAGTTTGAG–3') to primer SP3C (5'–GGAGTCACATGAGTAATCGC–3'); B) 1,134 b.p. from primer SP5B (5'–CAGGATAATGAGGTTGTGC–3') to SP3B (5'–ATACAACTTTCAGCAGGAGAC–3'); and C) 1,311 b.p. from SP5A (5'–CAAGGAGTTTATGTGTGGC–3') to SP3A (5'–CCAATTCATGAACCCG–3'). Overhanging A residues were added using Taq polymerase (Perkin Elmer) and the fragments were cloned into pGEM–T (Promega) or pCR2.1 (Invitrogen). DNA was purified using a SNAP mini–prep kit (Invitrogen) and sequenced on an ABI sequencer by the UCSF Biomolecular Resource Center.

ß–Galactosidase detection and immunostaining

For detection of ß-Galactosidase, a PAP pen (Research Products International) was used to draw hydrophobic lines on poly-lysine coated slides, creating a single well (12 wells were created on each slide for mutant screens). 1.5µl of 25% glutaraldehyde (Sigma) was added to each well, followed by 15µl of worms that had been rinsed several times in dH_2O + .01% Triton X–100 (Sigma). After 3 minutes of fixation an 18 mm^2 coverslip was placed over the worms, excess liquid was removed by aspiration, and the slides were frozen on dry ice. After 5 or more minutes, coverslips were pried off with a razor blade and the frozen slides were dipped in room temperature Acetone (Sigma) for 1 minute. Slides were air dried and then stained overnight using

a variation of the ß-galactosidase stain solution described by Fire et al. (1990), with 10 mM $MgCl_2$.

Anti–MAB–5 staining was done as described by Salser and Kenyon (1996).

Anti–LIN–39 staining and staining using the monoclonal antibody MH27 was done as described by Maloof and Kenyon (1998).

In all cases the nuclear stain DAPI was also included to assist in cellular identification.

RESULTS

polyray–1 is required to prevent ectopic Hox expression

To investigate the mechanisms that restrict expression of *mab–5* to the posterior, we performed a screen for mutations that disrupted the correct expression pattern of a *mab–5–lacZ* reporter construct. We identified a mutation, *mu38*, that caused widespread ectopic expression of the *mab–5* reporter in anterior V cells, in QR, and in a variety of other cell types (Fig. 2.1 A–H). This mutation defined a new gene, *polyray–1* (*pry–1*). Recently a second, slightly weaker allele, *nc1*, has been isolated in an independent screen (S. Takagi, personal communication). Both alleles of *pry–1* are recessive, suggesting that wild–type *pry–1* is required to keep *mab–5* expression off in

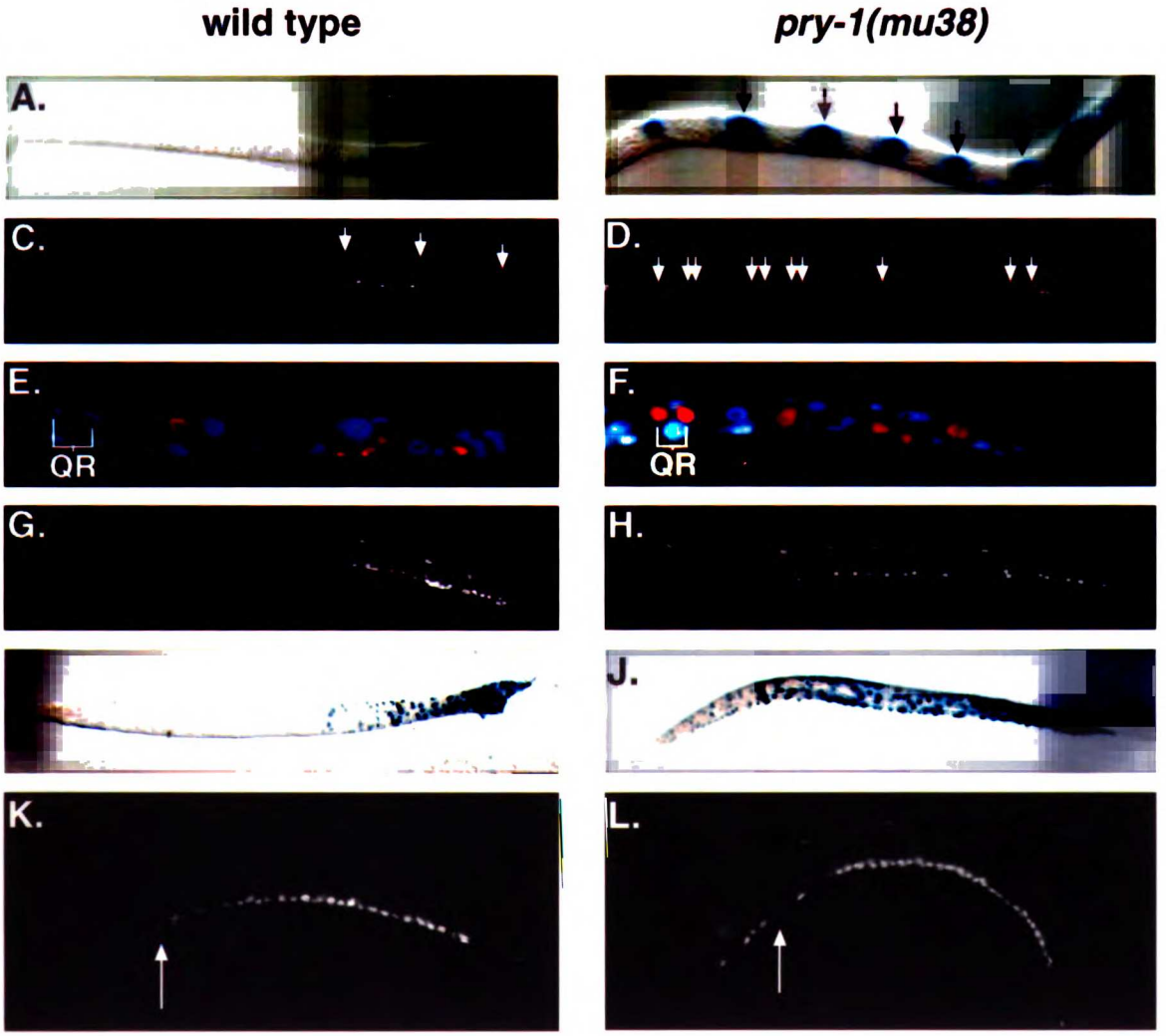

Figure 2.1. *pry-1* is required to keep Hox genes repressed
Anterior is left, ventral is down.
(A) Wild-type L1 worm carrying *muIs3(mab-5-LacZ)*. V6 (arrow) is staining
(B) *pry-1(mu38) ; muIs3(mab-5-LacZ)* L1, showing ectopic expression in the anterior V cells (arrows).
(C) Wild-type L2. Expression is limited to the posterior V cells (arrows).
(D) pry-1(mu38) L2. Expression is seen in the anterior and posterior V cells (arrows).
(E) and (F) are false-color, composite images of animals stained with anti-MAB-5 antibodies (red) and DAPI (blue).
(E) Wild-type L1, showing that *mab-5* is not expressed in QR.
(F) *pry-1(mu38)* L1. Ectopic mab-5 expression can be seen in QR.
(G) Wild-type expression of *mab-5* is limited to the posterior ventral cord cells (bottom of the worm).
(H) *pry-1(mu38)* extends *mab-5* expression into the anterior ventral cord.
(I) Wild type L4 male carrying *muIs13(egl-5-LacZ)*. Expression is limited to the posterior.
(J) *pry-1(mu38) ; muIs13(egl-5-LacZ)* L4 male. Expression is seen throughout.
(K) Wild type stained with anti-LIN-39 antibodies. Ventral cord staining stops at thelateral ganglion (arrow; identified by DAPI staining, not shown).
(L) *pry-1(mu38)* stained with anti-LIN-39 antibodies. Ventral cord staining extends past the beginning of the lateral ganglion (arrow).

the anterior V cells and in QR, and that the mutations reduce gene activity. To determine if *pry–1* is required for the regulation of other Hox genes, we examined the effect of *pry–1(mu38)* on the expression of *egl–5* and *lin–39*. *egl-5* is normally expressed in and patterns the tail of the worm, but *pry–1(mu38)* causes ectopic anterior expression of *egl–5–LacZ* (Fig. 2.1I, J; (Chisholm, 1991; Wang et al., 1993). Similarly, expression of *lin–39*, the gene which normally specifies cell fates in the mid–body of the worm (Clark et al., 1993; Wang et al., 1993); Maloof and Kenyon, 1998), is expanded anteriorly (Fig. 2.1K, L). Taken together these results show that *pry–1* functions as a general repressor that restricts Hox gene expression to the correct domains.

Initial expression of *mab–5* and *egl–5* is normal in *pry–1(mu38)*

Curiously, young *pry–1(mu38)* worms have relatively normal patterns of *mab–5* and *egl–5* expression (Fig. 2.2A, B). To determine the time course of ectopic *mab–5* expression, we examined expression at 4 hour intervals, starting at hatching (Fig. 2.2 C). We found that ectopic expression is first seen in the V5.p cell. Subsequently, ectopic *mab–5* expression appears in most of the Vn.ppp cells. It appears that in these cells, wild–type *pry–1* does not function to set the initial anterior limits of expression, but rather is required after the initial patterns are set to prevent ectopic expression of these genes. Thus *pry–1* may perform a function analogous to the *Polycomb*–group genes of *Drosophila*.

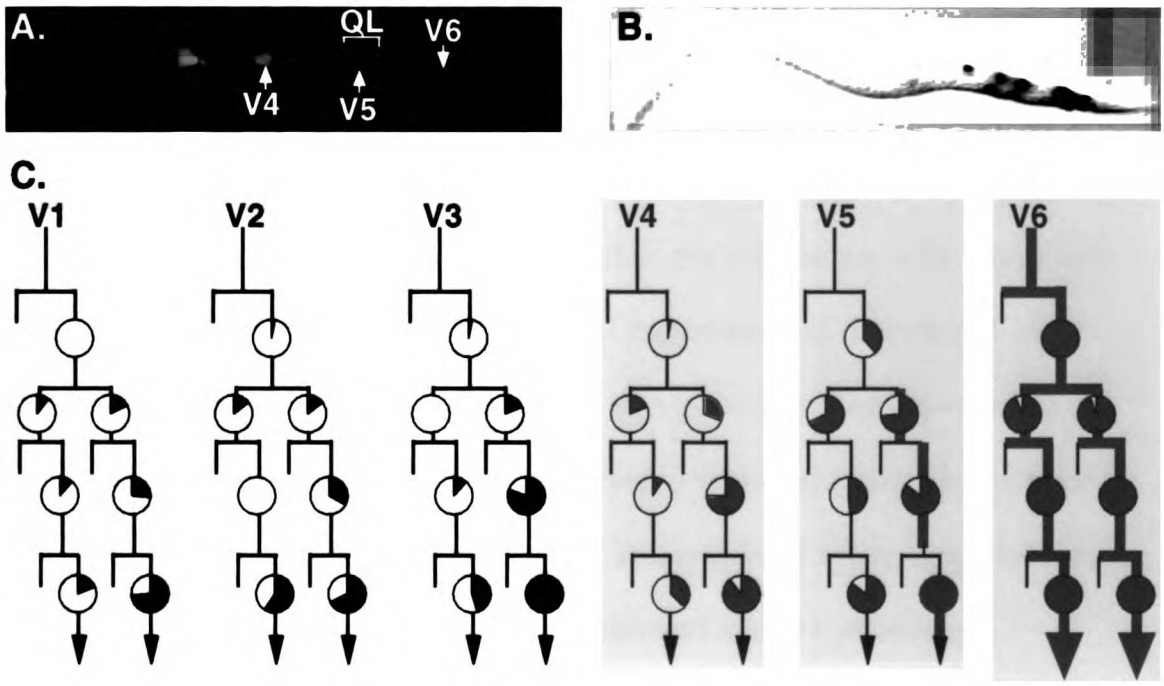

Figure 2.2. *mab-5* and *egl-5* are not ectopically expressed in young *pry-1(mu38)* animals.
(A) Young (L1) *pry-1(mu38)* animal stained with anti-MAB-5(red) and DAPI (blue) (false color composite image). *mab-5* is not yet ectopically expressed.
(B) Young (L1) *pry-1(mu38); egl-5-LacZ* animal stained with X-Gal. Ectopic expression of *egl-5-LacZ* has not yet begun.
(C) V cell lineage diagram showing timecourse of ectopic *mab-5* expression in *pry-1(mu38)*. Pie charts indicate the fraction of cells found to stain (blue), at each point in the lineage, in *pry-1(mu38)* mutants. Blue lines indicate cells that express *mab-5* in wild type; black lines indicate cells that do not express *mab-5*.

The *mu63* mutation suppress the *pry–1* phenotype

The ectopic expression of Hox genes that results from the *pry–1(mu38)* mutation causes the worms to be unhealthy: the worms are scrawny, often herniated, and uncoordinated. The *mu63* mutation was discovered as a spontaneous suppressor of *pry–1(mu38)* in a population of *pry–1(mu38)* worms that appeared healthy. To test whether or not the suppressor was extragenic, we outcrossed the suppressed strain to wild type. Approximately 1/4 of the F2 progeny from the outcross showed a *pry–1* phenotype, demonstrating that the suppressor was not linked to *pry–1* and therefore was extragenic.

***mu63* is an allele of *bar–1*, a *ß–catenin/armadillo* related gene**

We mapped *mu63* to a small region of the X chromosome between the cloned genes *unc–6* and *dpy–7*. Interestingly, the gene *bar–1*, a ß–catenin/*armadillo* homolog, mapped to the same interval (Eisenmann et al, in preparation). Mutations in *bar–1* were identified on the basis of a defect in vulval development suggestive of Hox gene mis–regulation. For this reason we hypothesized that *mu63* might be an allele of *bar–1*. Indeed we found that *mu63* failed to complement *bar–1(ga80)*, supporting the idea that *mu63* is an allele of *bar–1*. To confirm this, we sequenced *bar–1* cDNA from *mu63* and found a missense mutation in the first *arm* repeat which changed conserved

Leu-130 to Phe. Subsequently, a number of additional alleles of *bar–1* have been identified in a screen for mutations affecting Q cell migration (Mary Sym, Queelim Ch'ng, and C.K., unpublished). One of these new alleles, *bar–1(mu349)*, causes Gln-147 (also in the first *arm* repeat) to be changed to a stop codon. *mu349* is therefore likely to be a strong loss of function or null allele. To determine whether the ability of *bar–1* to suppress *pry–1* was allele specific, we constructed a *pry–1(mu38); bar–1(mu349)* double mutant strain. We found that the putative null allele of *bar–1* was also able to suppress *pry–1(mu38)*, demonstrating that wild–type *bar–1* is required for manifestation of the *pry–1* mutant phenotype.

bar–1* is required for ectopic Hox expression in *pry–1(mu38)

The *Drosophila bar–1* homolog *armadillo* in is known to act as a transcriptional coactivator (van de Wetering et al., 1997), suggesting two possible ways that *bar–1* mutations might act to suppress *pry–1(mu38)*. Wild–type *bar–1* could function as an activator to promote Hox gene expression; in this case, mutations in *bar–1* would suppress *pry–1(mu38)* by preventing ectopic Hox expression. Alternatively, *bar–1* might act combinatorially as an activator with the ectopically expressed Hox genes, thereby allowing them to carry out their functions. To distinguish between these possibilities, we examined expression of *mab–5* in *pry–1(mu38); bar–1(mu63)* and in *pry–1(mu38); bar–1(mu349)* strains. We found that

mab–5 was no longer ectopically expressed in either strain (Fig 2.3 and data not shown). Therefore, *bar–1* is required for the ectopic *mab-5* expression normally seen in *pry–1(mu38)* worms, and thus *bar–1* mutations likely suppress the *pry–1* phenotype by preventing ectopic Hox gene expression.

Because *bar–1* is required for ectopic *mab-5* expression in *pry–1* mutants, we wondered if *bar–1* was also required for Hox expression in otherwise wild–type worms. We found that in *bar–1(mu63)* worms, *mab–5* is expressed only weakly in QL, and that in the stronger *bar–1(mu349)* allele *mab–5* is not expressed at all in QL (Table 2.1 and data not shown). Curiously, although *bar–1* is required for the ectopic expression of *mab–5* in the V cells of *pry–1(mu38)* mutants, it is not required for wild–type expression in the V cells, suggesting that *bar–1* does not normally activate *mab–5* in these cells. It also has been shown that *bar–1* is required to maintain *lin–39* expression in the ventral epidermal cells (Eisenmann et al., in preparation). Thus, while *pry–1* normally functions to keep Hox genes off, *bar–1* functions in an opposite manner to activate or maintain Hox gene expression.

bar–1 and _pry–1_ function in a conserved Wnt pathway to control the direction of Q cell descendant migration

mab–5 is normally expressed in QL where it is required to specify that the descendants of QL migrate and remain in the posterior. The *egl–20*, *lin–17*, *mig–1*, and *mig–5* genes have all been reported to be required for correct

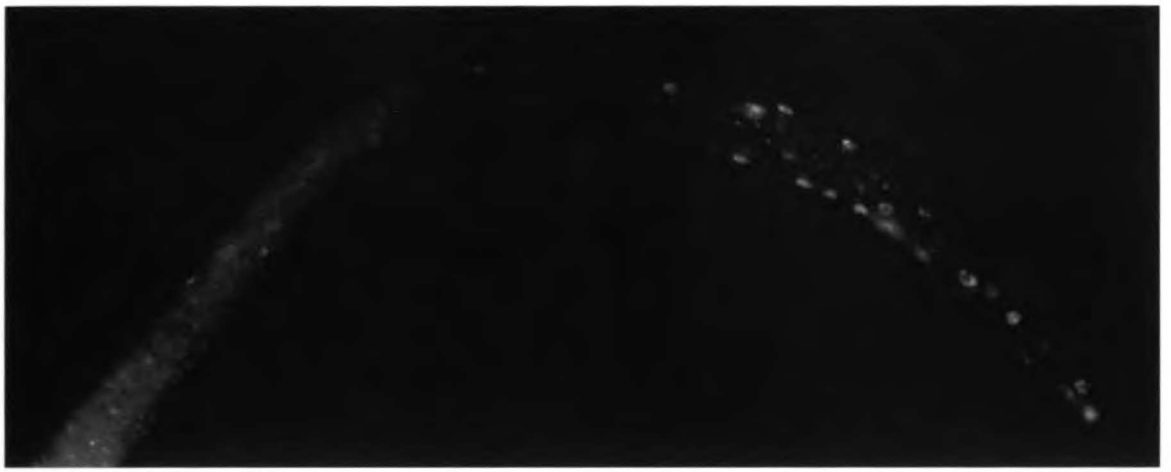

Figure 2.3. *bar-1(mu349)* is required for ectopic *mab-5* expression in *pry-1(mu38)* mutants. *pry-1(mu38); bar-1(mu349)* mutant animal stained with anti-MAB-5 antibodies. Staining is limited to the posterior (compare to fig. 1 D).

mab–5 expression in QL and for the subsequent migration of the QL descendants. Cloning of these genes has revealed that they encode components of a Wnt signaling cascade, demonstrating the importance of Wnt signals for switching on *mab–5* in QL. Since two different Wnt signaling pathways have been described in *Drosophila*, it was not clear which downstream signaling components would be involved in directing Q cell migration. We have found that in *bar–1* mutant worms *mab–5* is not expressed in QL, and, as a result, the QL descendants migrate anteriorly (Table 2.1). This establishes that a Wnt signaling pathway with components conserved from the Wnt signal to an *armadillo*-related transactivator functions to switch on *mab–5* in QL.

mab–5 is normally kept off in QR, and as a result the descendants of QR migrate into the anterior. In contrast to the large number of mutations that affect QL, only one mutation has previously been described that, without affecting the initial migration of QR, causes ectopic activation of *mab–5* in that cell: a gain–of–function promoter mutation in the *mab–5* gene itself. *pry–1(mu38)* also causes ectopic expression of *mab–5* in QR, and as a result the QR descendants stay in the posterior (Fig 2.1 F and Table 2.1). The QR migration phenotype is due to ectopic *mab–5;* in the *pry–1(mu38); mab–5(e2088)* double mutant, the QR descendants migrate to the anterior (Table 2.1). Thus *pry–1* represents a new class of gene involved in this process, one that is required in wild–type worms to repress *mab–5* in QR.

Table 2.1. *mab–5* expression in Q and final position of the Q descendants

Genotype	% QL expressing *mab–5*	% QL descendants in posterior	% QR expressing *mab–5*	% QR descendants in posterior
wild type	100	100	0	0
pry–1(mu38)	100	100	46	81
pry–1(nc1)	n.d.	100	n.d.	40
mab–5(e2088)	n.a.	0	n.a.	0
pry–1(mu38); mab–5(e2088)	n.a.	0	n.a.	0
bar–1(mu349)	0	0	n.d.	0
pry–1(mu38); bar–1(mu349)	0	0	0	0
mig–14(mu71)	0	0	n.d.	0
pry–1(mu38); mig–14(mu71)	n.d.	100	n.d.	100
egl–20(n585)	0	0	n.d.	0
pry–1(mu38); egl–20(n585)	63	88	70	71
mig–1(e1787)	28	15	n.d.	0
mig–1(e1787); pry–1(mu38)	94	100	73	82

mig–14, *egl–20*, and mig–1 single mutant data from Harris et al., 1996. Ten or more animals were scored for *mab–5* expression; twenty–five or more animals were scored for Q descendant final position. QL and QR were not scored for *mab–5* expression unless they had migrated over V5 or V4, respectively. The *mab–5* independent migration, which is not affected in these mutants, causes the QR descendants to be anteriorly displaced relative to the QL descendants. For this reason, the QR descendants were scored as being in the posterior when they were adjacent to or posterior to V3.p, whereas the QL descendants were scored as being in the posterior when they were posterior of V4.p. n.d., no data; n.a., not applicable.

The finding that *bar–1* is epistatic to *pry–1* demonstrates that *pry–1* functions upstream of or in parallel to this *armadillo* homolog. Therefore Wild-type *pry-1* likely acts to negatively regulate the ability of *bar-1* to activate expression of Hox genes, suggesting that *pry–1* functions as a negative regulator of Wnt signal transduction. To investigate further where *pry–1* acts in the Wnt pathway, we constructed strains that were mutant for *pry–1* and either *egl–20 (Wnt)* or *mig–1 (frizzled)*. We also constructed a double mutant between *pry–1(mu38)* and *mig–14(mu71)* (also known as *mom–3)*, a gene required in the Wnt signaling cell for the production of signal. In *egl–20, mig–1,* and *mig–14* mutants, *mab–5* fails to come on in QL; however, in worms also carrying a *pry–1(mu38)* mutation, *mab–5* is switched on in both QL and QR (Table 2.1) This demonstrates that *pry–1* is epistatic to all three of these genes, and suggests that *pry–1* acts downstream of *egl–20, mig–1,* and *mig–14* , but upstream of *bar–1* to negatively regulate Wnt signal transduction.

***pry–1* is required for proper V cell patterning**

The ectopic expression of *mab–5* in anterior V cells caused by *pry–1(mu38)* has at least two phenotypic consequences. First, the descendants of V5.pa normally make a sensory structure known as a postderid; however, if *mab–5* is ectopically expressed in V5.pa, then no postderid is made (Salser and Kenyon, 1996). Similarly, *pry–1* mutants often do not make a postderid, and

this effect is due to ectopic *mab–5* (Table 2.2A). Second, in males *mab–5* specifies that the V cells differentiate to make sensory rays instead of alae. In *pry–1* mutants the V cells never make alae and instead adopt ray–like fates (Fig. 2.4; Table 2.2B). The ray phenotype is also caused by ectopic expression of *mab–5*, because in *pry–1(mu38); mab–5(e2088)* double mutants, almost all of the V cells adopt alae fates (Table 2.2B). Thus, in wild–type worms, *pry–1* is required to restrict *mab–5* expression to the posterior V cells so that the postderid and alae/ray fates can be specified correctly.

As mentioned above, even though *bar–1* and *pry–1* have opposite effects on *mab–5* expression in the Q cells, we found that *mab–5* is expressed normally in the V cells of *bar–1* mutant worms. In addition, the V rays are made almost as frequently as in wild–type: nine out of ten *bar–1(ga80)* males examined had six V rays, as do wild type; the remaining animal had 5 rays. This suggests either that *bar–1* is not a major component of the machinery used to activate *mab–5* expression in wild–type V cells, or that it is redundant with other activators of *mab-5*. *bar–1* can clearly function in the majority of the V cells, since it is required for the ectopic expression of *mab–5*, the extra rays, and the loss of the postderid caused by *pry–1* mutations: these *pry–1* phenotypes are fully suppressed in *pry–1; bar–1* double mutants (Table 2.2). This suggests that the downstream components of the Wnt signaling pathway are functional in the V cells, but that Wnt signaling is not required during wild–type development to activate *mab–5* expression.

Table 2.2 Changes in V cell fate specification

Genotype	(A) % with postderid	(B) Alae/ray fates	
		Extent of V cell derived Alae	Average # of anterior V cell derived rays
wild type	100	V1–V5	0
pry–1(mu38)	75	none	22.2
bar-1(ga80)	100	V1-V5	0
mab–5(e2088)	100	V1–V6	0
pry–1(mu38); mab–5(e2088)	100	weak V1–V6	0.6
pry–1(mu38); bar–1(mu349)	100	V1–V5	0
wild type, V6 ablated	0	n.a.	n.a.
bar–1(ga80) V6 ablated	88	n.a.	n.a.

Ten or more animals were scored for each genotype. All strains included *him–5(e1490)* to increase the frequency of males. Rays from anterior V cells were assayed in *pry–1(mu38)* by using the monoclonal antibody MH27 to outline the ray socket cell in combination with DAPI staining to identify ray cell group nuclei (Salser and Kenyon, 1996). Only ray cell groups anterior to the anus/intestine junction were scored, allowing identification of V1–V4 derived, and some V5 derived, rays. For all other genotypes, anterior rays were scored by looking for papillae (ray tips) associated with gaps in alae.

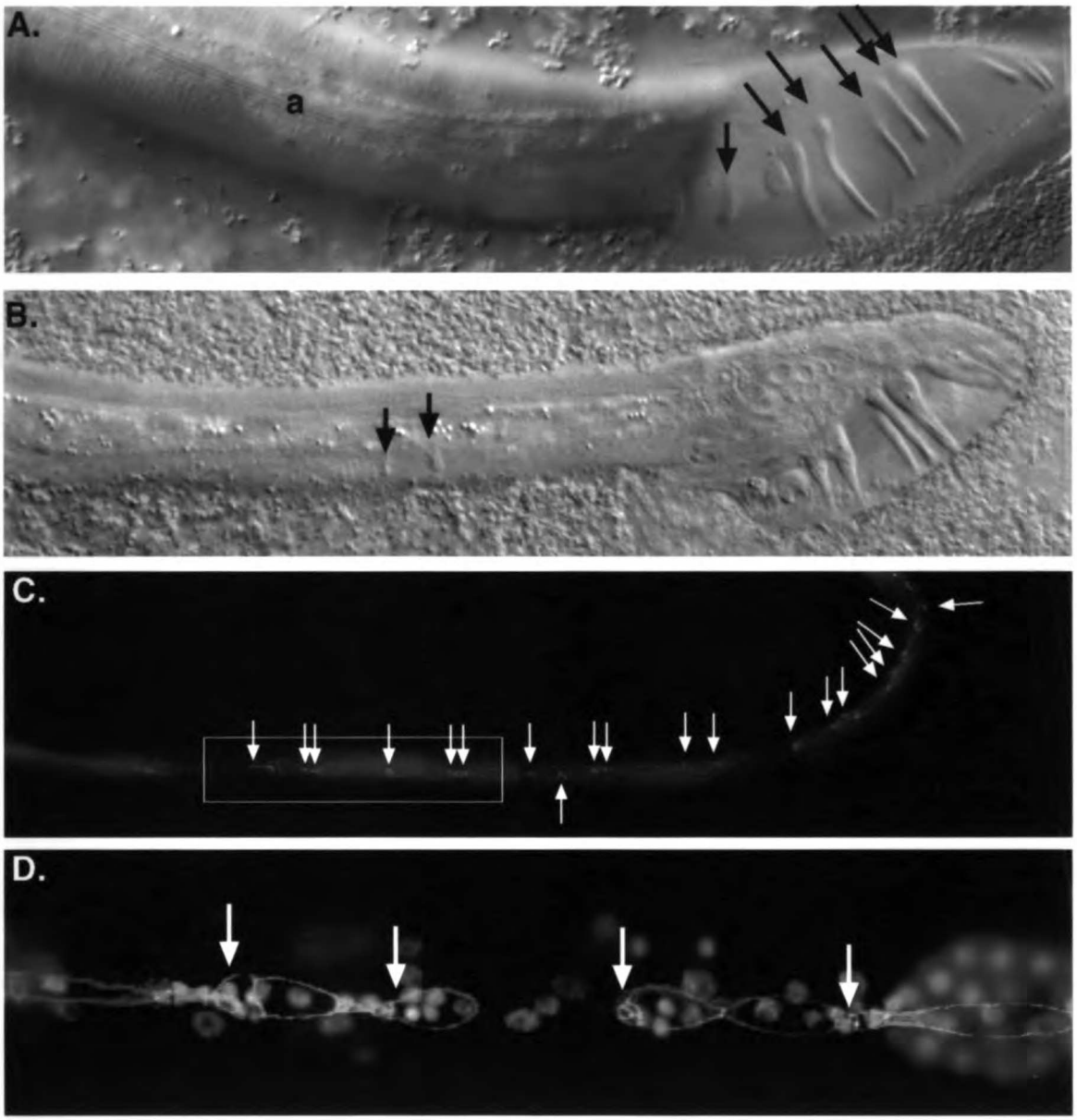

Figure 2.4. *pry-1(mu38)* causes ectopic rays to be made.
(A) Wild-type male tail showing rays (arrows) and alae (a). Nomarski image.
(B) *pry-1(mu38)* male tail. The alae have been replaced with ectopic rays (arrows). Rays in the body do not extend away from the cuticle, but can be identified by their papillae, or ray tips.
(C) L4, *pry-1(mu38)* male, stained with MH27 monoclonal antibody. At this stage, each of the 3 cells that make up the ray cell group are outlined by the antibody (arrows).
(D) Enlargment of the region boxed in (C). False color composite image showing MH27 (red) and DAPI (blue). The ray cell groups can be identifed both by MH27 outlines and by the clustered small nuclei (arrows).

Why are the V cells able to respond to activation of the Wnt pathway if it is not used during wild-type development? The Wnt pathway may be used to compensate for perturbation during development. (Sulston and White, 1980) observed that if V6 is ablated with a laser microbeam, then V5 adopts a V6-like fate; instead of making a postderid and single ray, it instead makes 5 rays and no postderid. Ablation causes constitutive *mab-5* expression in V5 which is both necessary and sufficient for the transformation to occur. It has recently been found that the *egl-20 Wnt* and *lin-17 frizzled* genes are required both for constitutive *mab-5* expression in V5 and its transformation (Craig Hunter, Jeanne Harris, and C. K., in preparation). To test whether *bar-1* is involved in this compensation process, we ablated V6 in *bar-1* animals. We found that unlike in wild-type, the postderid is still made after V6 ablation in *bar-1(mu349)* mutants (Table 2.2A) Since *mab-5* is required for the postderid to seam cell fate transformation, this result suggests that like *egl-20* and *lin-17, bar-1* is required to activate *mab-5* expression in V5 as a response to V6 ablation.

DISCUSSION

Our results demonstrate that the *pry-1* and *bar-1* genes function in a conserved Wnt signaling pathway to control the post-embryonic expression of multiple Hox genes in multiple tissues. Proper regulation of this pathway is critical: activation is required to promote Hox gene expression in selected

cells to achieve proper patterning, and inhibition is required to prevent widespread ectopic activation of Hox genes and the resulting homeotic transformations. Our results show that Wnt signals are critical for patterning Hox gene expression in *C. elegans*. We also have found that *bar–1* is epistatic to *pry–1*. Together with the expression data, this suggests that in wild–type worms *pry–1* inhibits *bar–1* activity and thereby represses Hox genes expression and that Hox genes can be activated by inhibiting *pry–1* activity.

A conserved Wnt signaling system acts as an on/off switch for *mab–5* in the Q cells

Recent reports have demonstrated that *egl–20, lin–17, mig–1,* and *mig–5* encode upstream components of a conserved Wnt signaling cascade, and are important for initiating *mab–5* expression in QL. The *Drosophila* homologs of these genes-*wingless, frizzled,* and *disheveled*-are known to activate two different downstream pathways: the first one is known to be well conserved and includes the *ß–catenin/armadillo* and *dTcf/pangolin* genes, while the second is a less well defined pathway required for tissue polarity. Our finding that the *ß–catenin/armadillo* homolog *bar–1* is required to turn on *mab–5* in QL shows that the *C. elegans egl–20, lin–17,* and *mig–1* genes activate a pathway homologous to the first described above. The fact that *egl–20, lin–17,* and *mig–1* mutants share some phenotypes that *bar–1* mutants do not (for example a defect in the migration of the HSN neuron [Harris, 1996 #220; data

not shown]) raises the possibility that they may activate a second type of signaling pathway in other cells.

One of the most interesting aspects of Q cell migration is the fact that in spite of being born in bilaterally symmetric A/P positions, QL and QR, and their descendants, migrate in opposite directions. One critical difference between these cells is the expression of *mab–5* in QL but not in QR, and that this difference is necessary and sufficient for controlling the behavior of the Q descendants. Why is it that *mab–5* is switched on in QL but not in QR? The observation that *pry–1* is required to keep *mab–5* off in QR demonstrates that QR is sensitive to activation of the Wnt pathway and has all of the necessary components downstream of *pry–1*. Therefore, we propose that the critical difference between QL and QR with regards to *mab–5* expression is in activation of or transduction through the Wnt pathway upstream of *pry–1*.

The fact that QL migrates towards the posterior and QR towards the anterior before *mab–5* expression begins may be important for differential activation of the Wnt pathway. If *egl–20/Wnt* is expressed only in the posterior, then QL's posterior migration could bring it into contact with the signal and cause activation of *mab–5*, whereas the anterior migration of QR would move it away from the signal and leave *mab–5* off. Models that do not depend on the initial migration of QL and QR are also possible. For example, *egl–20* expression could be limited to the left side of the animal. Alternatively, the *lin–17* and/or *mig–1* receptors, or other components

downstream of *Wnt*, may be differentially expressed on the two sides of the worm. Localization of these molecules should provide exciting information allowing the mechanism of specific *mab–5* activation in QL to be elucidated.

pry–1 functions as a general repressor of Hox gene activation

pry–1 is required in many tissues to keep Hox genes off: it represses *egl–5* in epidermal cells, *lin–39* and *mab–5* in ventral cord neurons, and *mab–5* in the Q cells and in the V cell descendants. Interestingly, the initial expression patterns of both *mab–5* and *egl–5* in the epidermis is largely normal; dramatic ectopic expression is not seen until many hours after hatching. For this reason, *pry–1* phenotypically resembles genes of the *Polycomb*–group of *Drosophila*, which are thought to repress Hox gene expression by affecting chromatin dynamics. However, we have found that *pry–1* mutations can be suppressed by mutations in the *ß–catenin/Armadillo* homolog *bar–1*, suggesting that *pry–1* does not affect chromatin structure, but rather functions to inhibit transcriptional activation by *bar–1*. This idea is further supported by the finding that *pry–1* is epistatic to a number of genes encoding members of a Wnt signaling cascade. Although the molecular identity of *pry–1* is not known, it may encode a *C. elegans* homolog of a Wnt pathway regulator such as *zeste–white 3* (Peifer et al., 1994; Siegfried et al., 1992), Axin (Zeng et al., 1997), or APC (Munemitsu et al., 1995; Polakis, 1997; Rubinfeld et al., 1996).

The *Drosophila Wnt* homolog *wingless(wg)* is known to control

transcription of *Ubx* in the developing midgut, but in contrast to our results, the *Wnt* pathway in *Drosophila* is not known to play a major role in regulating Hox expression. The Wnt pathway may be more important for regulating *Drosophila* Hox genes than has been realized, this phenotype may have been missed due to other more dramatic consequences of mutations in the Wnt pathway. If so, it is possible that some of the uncloned *Polycomb*–group members could in fact be regulators of Wnt signal transduction. Alternatively, the differences in the importance of Wnt signaling for Hox gene expression may reflect evolutionary differences between worms and flies. For this reason it will be interesting to learn whether the Wnt pathway is a relatively minor or major regulator of Hox gene expression in other species.

The need for specificity

bar–1 and *pry–1* function quite differently in different cell types. Not only do the Hox genes under their control vary from tissue to tissue, but the precise role that *bar-1* and *pry-1* play during wild–type development varies as well. For example, in the Q cells *bar–1* and *pry–1* function as part of an on/off switch that keeps *mab–5* expression off in QR and initiates *mab-5* expression in QL. In the V cells, however, their primary role seems to be to prevent *mab–5* expression in the anterior; *pry–1* mutations cause ectopic *mab–5* expression, but loss of *bar–1* has little or no effect on *mab-5* expression or V

cell development. In contrast, *bar–1* is required in the Pn.p cells to maintain *lin–39* activation after expression has been initiated by other factors (Eisenmann et al., in preparation).

Cells also change their responsiveness to *bar-1* and *pry-1* temporally. For example, although *pry–1* mutations cause ectopic expression of *mab–5* in QR, this ectopic expression does not appear until the time that wild–type *mab-5* expression begins in QL, as if the Q cells are not competent to respond to activation of the Wnt pathway until that time. Similarly ectopic expression in the V5 lineage does not begin until late in the first larval stage. Taken together these observations suggest that other spatially and temporally regulated factors interact combinatorially with *bar–1* and *pry–1* to modulate and specify their actions. Identifying these factors, and understanding their interactions with *bar-1* and *pry-1* should provide interesting insights into the control of the Wnt pathway during development.

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

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.

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 sex-combs reduced (scr)* homolog, 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. 3.1A) (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. 3.1B) (Aroian

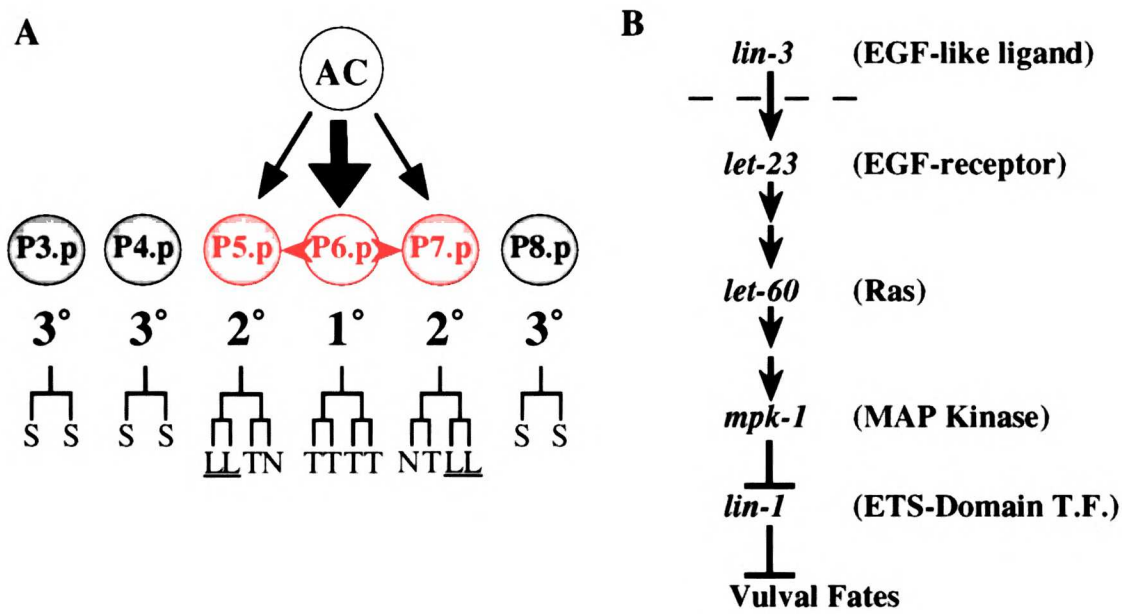


Figure 3.1. Model for Vulval Induction
(A) Schematic of the AC and VPCs at the time of induction, and lineage diagram of the VPCs after induction. Anterior is to the left, ventral is down. Each VPC can adopt one of three fates, distinguishable by lineage analysis and cell morphology. The graded AC signal (black arrows), along with lateral signals between the VPCs (green arrows) 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).

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).

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)* (JNM 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 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 MeOH, incubated at room temperature for 1 minute, rinsed 3X 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 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 MeOH for 3 minutes, rinsed 2X in TBSTwE, and then blocked for 30 minutes at 37°C. Larvae were incubated with primary antibody for 1–2 hours, washed 3 X 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 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 lineaged beginning with the Pn.px or Pn.pxx stage. VPC fate assignment was made as described by Katz et al. (1995).

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. 0–2 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 3. 1B, and *lin–39; lin–1* double mutants), we used the following program: 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 (times are given as hours:minutes).

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 3. 1C), or an 8 minute, 33°C heat pulse was added 2 or 3 hours after the 6th 31°C pulse (Table 3. 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 were, with 3 hrs separating each pulse.

To provide *lin–39* to males (Fig. 3.4B, 3.5B), 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 (Fig. 3.4D, 3.5C), 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.

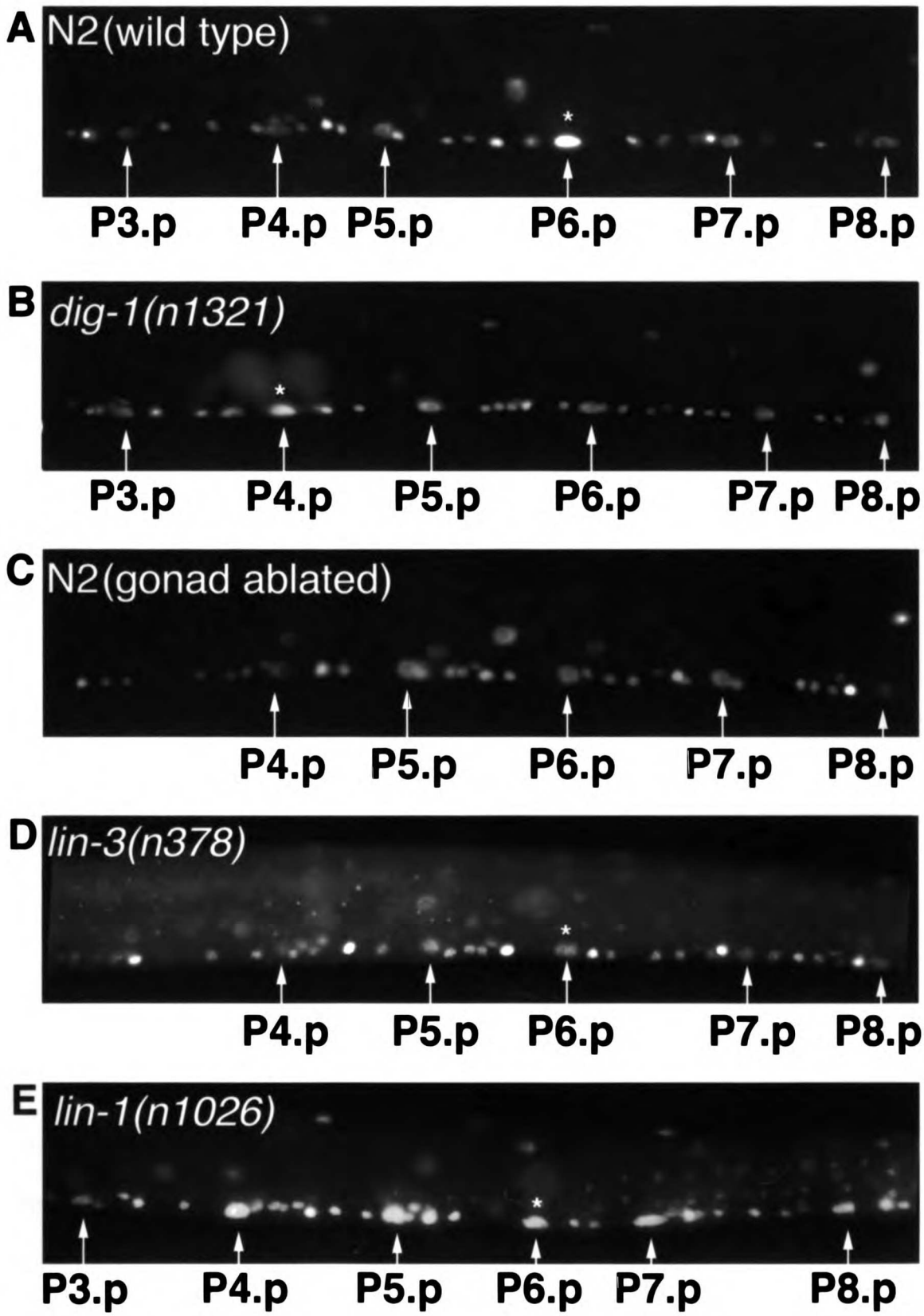
RESULTS

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. 3.2A). 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. 3.2B). Second, ablation of the cells that generate the AC abolished the peak of expression (Fig. 3.2C). Third, Vulvaless (Vul) mutations that reduce Ras signaling decreased peak levels of Hox gene expression (Fig. 3.2D), whereas Multivulva (Muv) mutations that ectopically activate the signaling pathway caused strong expression in all VPCs (Fig. 3.2E). 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

Figure 3.2. *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 maybe a Ras–independent mechanisms for biasing *lin–39* expression in the VPCs. * indicates approximate AC position.


A
N2(wild type)
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
B
dig-1(n1321)
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
C
N2(gonad ablated)
P4.p
P5.p
P6.p
P7.p
P8.p
D
lin-3(n378)
P4.p
P5.p
P6.p
P7.p
P8.p
E
lin-1(n1026)
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p

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 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 3. 1B, especially note P6.p descendants). Later and stronger heat pulses restored wild–type vulval development (Table 3. 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.

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

Table 3.1. Response of *lin-39(-)* animals to pulses of *hs-lin-39*

A. Wild type

	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
	SS	SS	LL TN	TTTT	NTLL	SS

B. *hs-lin-39* (low)

	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
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	UL LN	SS	UU UU	SS
11.	S	S	S	SS	LL UU	S
12.	S	S	S	SS	TLU U	S
13.	S	S	S	SS	TUU U	S
14.	S	S	S	SS	OO NU	S
15.	S	S	S	UU S	LL UU	S
16.	SS	SS	SS	S UL	SS	SS
17.	SS	SS	S TN	S LT	NT UU	SS
18.	S	S	S	NT LN	UU S	S
19.	S	S	S	TUUT	S	S
20.	S	S	SS	TUTN	NLU U	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	UU LL	SS	SS	S
23.	S	S	SS	SS	LLUU	S
24.	S	S	UU UU	SS	UU UU	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	LL UU	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	UU TN	TTTL	S	S
33.	S	SS	SS	NTTT	NUUU	S
34.	S	S	S	TTTL	NTLU	S
35.	S	S	S	TTTT	TL TU	S
36.	SS	SS	SS	TTTT	NTLU	SS
37.	D	D	S UU	TTTT	LL UU	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	UU LN	TTTT	NTUL	S
42.	S	SS	UL LN	TTTT	NLUU	SS

D. *hs-lin-39* (high)

43.	S	S	S	SS	LL LL	S
44.	SS	SS	SS	SS	LT LL	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	UU TN	TTTT	NT LU	SS
52.	SS	SS	UU TN	TTTT	NTLO	SS
53.	S	SS	UL TN	TTTT	NT UU	SS
54.	SS	SS	UL TN	TTTT	NT LS	SSS
55.	SS	SS	LO TN	TTTT	NTLL	SS
56.	SS	SS	LL TN	TTTT	NTLL	S
57.	SS	SS	LL TN	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° pulses.
(C) *lin–39(–); hs–lin–39* given seven 31° pulses.
(D) *lin–39(–); hs–lin–39* given six 31° pulses followed by a 33° pulse (see Materials and Methods for details).

See Figure 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)

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. 3.2E). 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 3. 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. 3.3A); 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

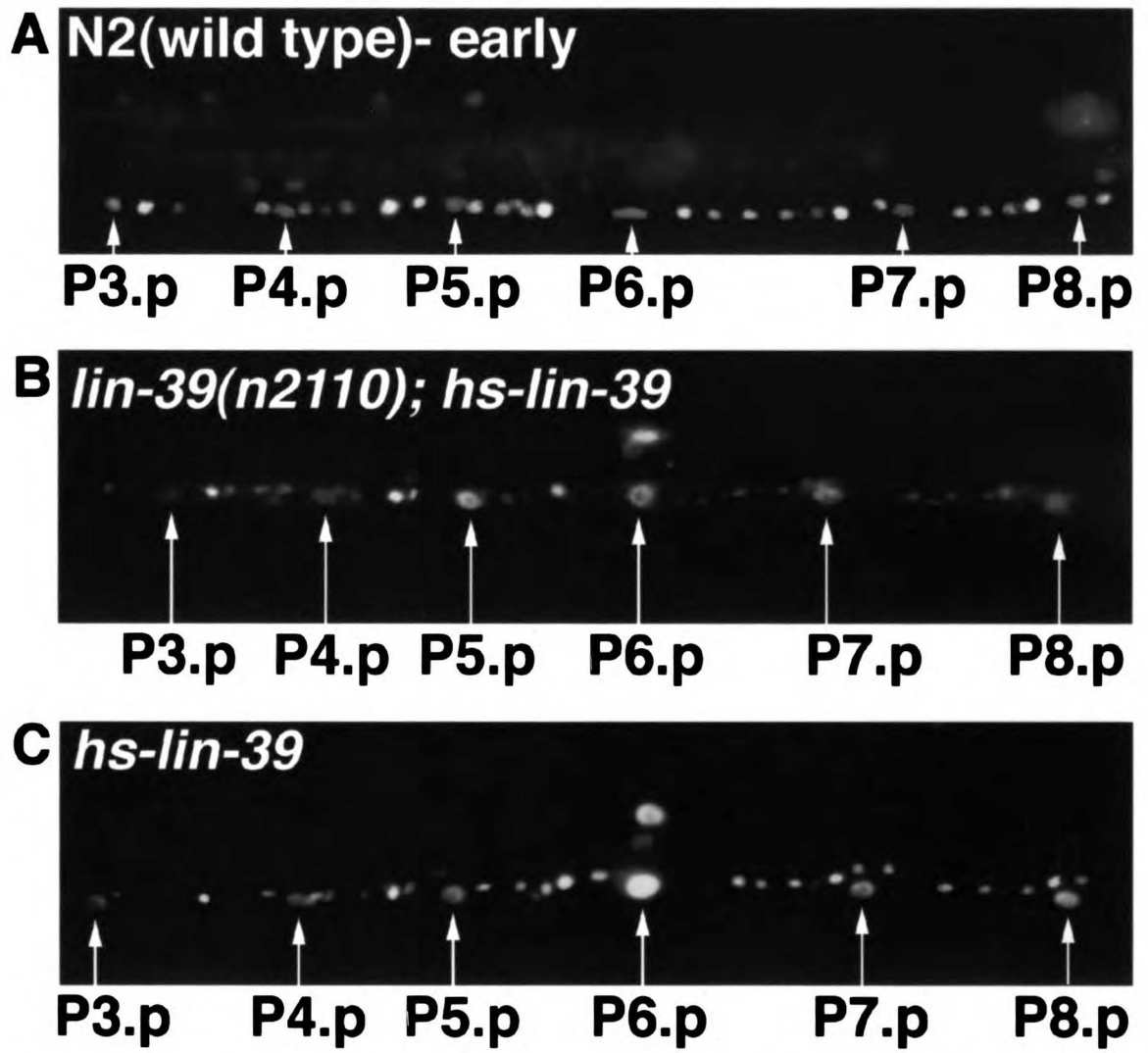


Figure 3.3 *lin–39* is required for its own up–regulation in response to Ras signaling (A) In wild–type larvae, *lin–39* is expressed uniformly at "basal" levels in the VPCs before vulval induction.
(B) *lin–39(n2110); hs–lin–39* mutant showing relatively uniform levels of expression in P(5–7).p, similar 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 *hs–lin–39* animal (otherwise wild–type) 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.

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. 3.3B; compare to *hs–lin–39* animals with *lin–39(+)* in Fig. 3.3C). The *n2110* mutation is a single base change in the 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**

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 in male 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. 3.4A, 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. 3.5A, 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. 3.5A, 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. 3.4B), induced some T

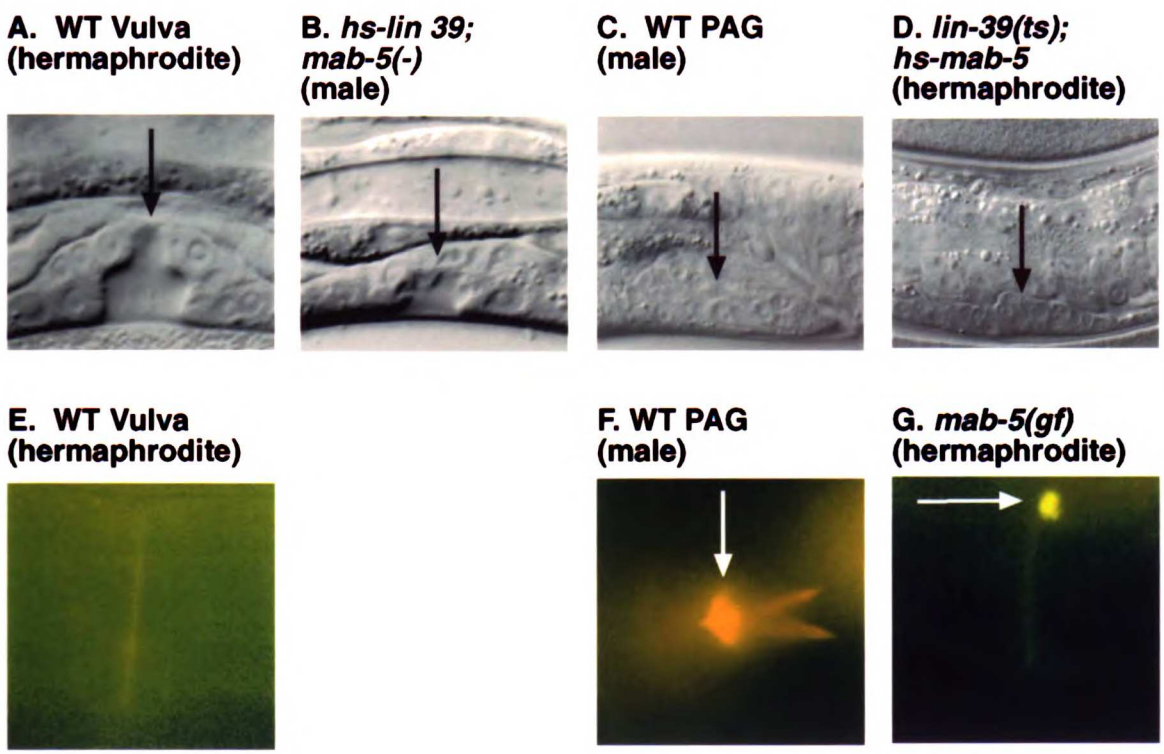


Figure 3.4. Hox genes play a role in specifying organ identity.
(A) Photograph of a wild–type hermaphrodite showing vulval invagination (arrow) during L4.
(B) Representative *mab–5(–)* male after *hs–lin–39*. Descendants of P10.p and P11.p formed an invagination (arrow) reminiscent of the invagination seen in the wild–type hermaphrodite vulva.
(C) Photograph of 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) Picture of 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 punctate autofluorescent regions 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.

Figure 3.5. Hox gene mis-expression causes vulval and PAG transformations. (A) Wild–type vulval lineage (see Fig. 1 and Table 1 for key). 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.
(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 a PAG-like fate. Adherence to the cuticle was not scored in these animals (so no cell 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) is adopted by P6.p in at least three of the *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 Mat. and Meth. 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 (bold) 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).

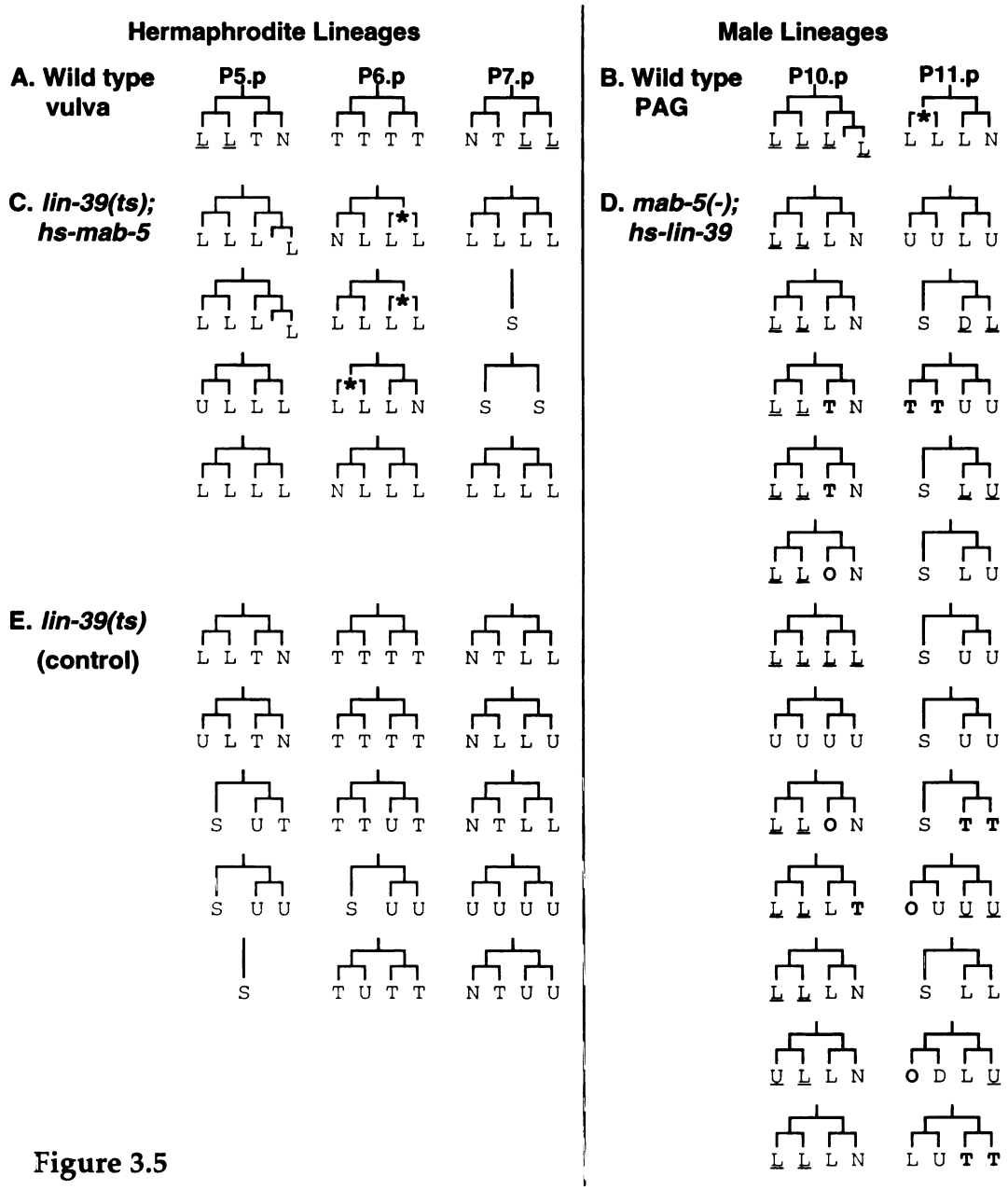

Hermaphrodite Lineages
Male Lineages
A. Wild type vulva
P5.p
P6.p
P7.p
L L T N
T T T T
N T L L
B. Wild type PAG
P10.p
P11.p
L L L L
L L L N
C. lin-39(ts); hs-mab-5
L L L L
N L L L
L L L L
L L L L
L L L L
S
U L L L
L L L N
S S
L L L L
N L L L
L L L L
D. mab-5(-); hs-lin-39
L L L N
U U L U
L L L N
S D L
L L T N
T T U U
L L T N
S L U
L L O N
S L U
E. lin-39(ts) (control)
L L T N
T T T T
N T L L
U L T N
T T T T
N L L U
S U T
T T U T
N T L L
S U U
S U U
U U U U
S
T U T T
N T U U
L L L L
S U U
U U U U
S U U
L L O N
S T T
L L L T
O U U U
L L L N
S L L
U L L N
O D L U
L L L N
L U T T

Figure 3.5

divisions characteristic of vulval cells, and caused detachment patterns reminiscent of those seen in the vulval secondary lineage (Fig. 3.5D). 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 under ultra-violet (u.v.) light (Link et al., 1988) (Fig. 3.4F) whereas the vulva is not (Fig. 3.4E); thus, autofluorescence can be used as 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. 3.4G; 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. Interestingly, 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 affect a 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. 3.4D). 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. 3.5C). 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. 3.5), and the other daughter generated a descendant that did not divide again (N in Fig. 3.5). Two animals had a P5.p granddaughter that underwent an additional division, giving a P5.p lineage identical to that

of a normal P10.p in the PAG. These division patterns are never seen in wild-type or control vulval lineages. Thus replacing *lin-39* with *mab–5* causes a transformation of vulval cells to PAG 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. In mice, expression of Hox genes in the hindgut is under control of *sonic hedgehog* (Roberts et al., 1995) and in *Drosophila, wingless* can control 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 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 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. 3.6A). 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 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. 3.6B). In this way, AC signaling limits gene activation to the central VPCs, and *lin–39* activity ensures that only vulva–specific genes

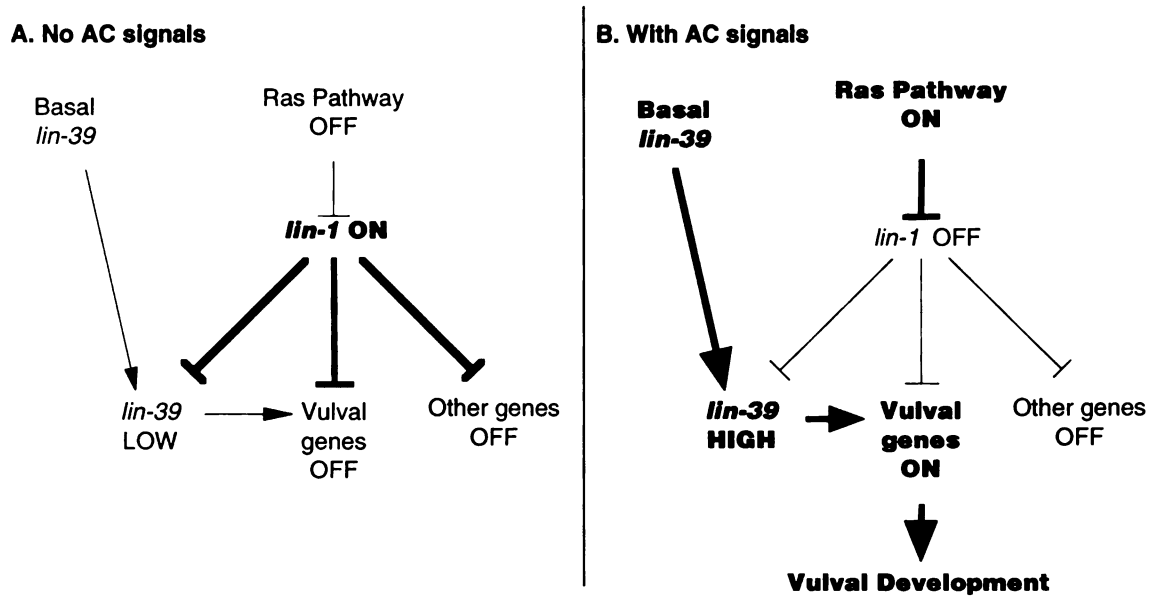


Figure 3.6. Model for vulval induction, incoroprating *lin-39*.
(A) In the absence of AC signals, *lin–1* is active and represses a number of genes including *lin–39* and vulval genes.
(B) AC signals inactivate *lin–1*, resulting in an increase in *lin–39* expression and enabling the activation of vulval genes by *lin–39*. Basal *lin–39* is required for up–regulation of *lin–39* expression in response to Ras signaling.

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 limit 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.

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Chapter Four: Concluding Remarks

In *C. elegans*, and probably all metazoans, controlling the spatial and temporal expression patterns of Hox genes is critical for achieving correct patterning along the anteroposterior (A/P) axis. It is important that expression of each Hox gene is initiated in and restricted to the appropriate A/P region. Furthermore, within their domains of action, intricate changes in the patterns of Hox gene expression are required to specify the development of specific structures. In spite of the fundamental homeotic transformations caused by defects in Hox gene expression or function, how Hox gene expression is regulated in most organisms remains poorly understood.

Although expression of a particular Hox gene within a cell often leads to autonomous determination of cell fate, many cell fate decisions are determined non-autonomously by extracellular signals that activate conserved signaling cascades. This thesis has focused on how the *C. elegans* Hox genes are controlled by two well conserved signal transduction cascades to correctly specify A/P fates. Rather than reiterate the discussions given in the individual chapters, the work in each chapter will be briefly summarized, and then some speculative ideas, questions, and future directions will be addressed.

The Wnt pathway plays a major role in controlling Hox expression in *C. elegans*.

Chapter two described a screen for genes affecting regulation of the Hox gene *mab-5*. This led to the discovery of *pry-1*, a gene required to prevent ectopic expression of at least three of the *C. elegans* Hox genes in multiple tissues during development. In contrast, *bar-1*, a spontaneous suppressor of *pry-1*, is required in multiple tissues to activate or maintain activation of the Hox genes. I originally viewed these genes as being required for maintenance of Hox expression and likely to be *Polycomb*-group and *trithorax*-group homologs; the realization that they function in a Wnt signaling cascade came as a surprise. Analysis of these genes has shown that the Wnt cascade that they are members of must be negatively regulated to prevent wide-spread ectopic Hox gene expression, and also must be specifically activated to promote fine-scale control of Hox gene expression. For example, the differential expression of *mab-5* in the bilaterally symmetric migratory neuroblasts QL and QR is achieved by selective activation of the Wnt pathway in QL but not in QR.

It is surprising that the Wnt pathway has the potential to control Hox genes to such a large degree in *C. elegans*. The dramatic nature of the changes seen in *pry-1* mutants is comparable to the changes caused by *Polycomb*-group mutations in *Drosophila* or by exogenous retinoids in vertebrates. Although *Ubx* has been found to be under the control of *wg* in the *Drosophila* midgut, the Wnt pathway is not considered to be a major Hox regulator in *Drosophila*

or vertebrates. Why the difference? It is possible that there really is not a difference; instead, activation of the Wnt pathway in other organisms may have such dramatic effects on other, non-Hox-related processes, that changes in Hox regulation have been missed. With this in mind, it would be interesting to see if there are major changes in Hox expression in *zeste-white 3* or *ß-catenin/armadillo* mutants in other species. It might be particularly informative to look in *Drosophila* at Hox expression in late mosaic clones where *zeste-white 3* or *ß-catenin/armadillo* had been removed.

Alternatively, the role of Wnt signaling in regulation of Hox genes may indeed be larger in *C. elegans*. There are many differences in the way *C. elegans* and *Drosophila* develop that could lead to different requirements for the Wnt pathway in Hox gene control. For example, due to the small size of *C. elegans* (at hatching the entire lateral epidermis is only ten cell diameters long, roughly the length of an embryonic *Drosophila* segment), a localized extracellular signal can impact a larger fraction of cells in the organism, perhaps favoring control of A/P information by proteins like Wnt. Another potentially large difference is the role of the *Polycomb*-group genes. In *Drosophila* these genes play a major role in keeping Hox genes repressed. In *C. elegans*, mutations in these genes have severe early embryonic consequences (Garvin et al., 1998), but so far appear to only have minor effects on Hox controlled A/P patterning (data not shown). If this is indeed the case, then the differential effect of constitutive Wnt pathway activation on Hox

gene expression in the two species may be due to differences in the extent to which hox genes are regulated by the *Polycomb*-group. In other words, constitutive activation of the Wnt pathway in*C. elegans* may have a dramatic effect on Hox gene expression because the *Polycomb* genes have a limited role in Hox regulation, whereas in *Drosophila,* the strong repressive actions of the *Polycomb*-group may prevent much Wnt driven Hox expression.

The changes in Hox expression observed in *pry-1* and *bar-1* mutants raise a number of specific questions about both Wnt signaling and Hox genes during patterning in *C. elegans.* Ectopic expression of *egl-5* was observed in the epidermis of *pry-1* mutants, but precise identification of these cells was difficult due to the roller mutation used as a coinjection marker with the *egl-5-LacZ* construct. Which cells are affected? Do Wnt signals normally control wild-type *egl-5* expression patterns in the tail epidermis? If so, what Wnt gene is responsible? *lin-44,* which encodes a Wnt molecule that is expressed in the tail (Herman et al., 1995); represents one good candidate. What are the patterning decisions being controlled? Similar questions are prompted by the ectopic *lin-39* expression seen in ventral cord neurons in *pry-1* mutants, and by the loss of *lin-39* expression in the VPCs caused by *bar-1* mutations.

One fascinating aspect of the *bar-1* and *pry-1* phenotypes is that different Hox genes are affected in different tissues and at different times. This implies that *bar-1* must act combinatorially with other spatially and

temporally controlled regulators to effect Hox expression. One way to identify such regulators would be to screen for *pry-1* suppressor and enhancer mutations. Suppressors should be easy to identify by looking for worms which are healthy and non-Unc. Enhancers could be identified by using a *mab-5-GFP* reporter construct to look either for earlier ectopic expression or for ectopic expression in the anterior branches of the V cell lineages. Similarly, one could use a *lin-39-GFP* construct to look for ectopic expression in the V cells. Enhancers may be difficult to identify because presumably they would make the already unhealthy *pry-1* strain even more so. This problem could be partially overcome by starting with the healthier *pry-1; mab-5* double mutant, and also by performing a clonal screen.

Another mystery highlighted by this work is the identity of the mechanism responsible for activating *mab-5* in the V5 lineage. *mab-5* expression is normally activated in V5.pp during the second larval stage. The ectopic *mab-5* expression seen in *pry-1(mu38)* animals shows that the V cell descendants are sensitive to activation by *bar-1* at this time, so we thought that Wnt signaling might be used here to activate *mab-5*. However, the V5 derived, *mab-5* dependent ray 1 is made in most *bar-1, egl-20, lin-17,* and *lin-44* mutants (chapter 2 and data not shown), suggesting that Wnt signaling is either not important or is a redundant component of *mab-5* activation in these cells. What is the nature of the activator? One possible way to screen for the activator is suggested by the *pal-1* mutant phenotype. In *pal-1(e2091)*

mutant males, *mab-5* is not turned on in V6, but expression is normal in the V5 lineage. This leads to a gap in the alae overlying the V5 descendants, followed by posterior alae over the V6 descendants. In this background it should be easy to identify mutations preventing *mab-5* expression in V5 descendants, by screening for animals in which the V5 derived gap in the alae is missing. It might be wise to perform this screen in a *bar-1(-)* background in case Wnt signaling is redundant with other activators. Finally this screen should be done clonally or in a *tra-1(e1488)* background (in which worms have male bodies but hermaphrodite gonads), since *pal-1(e2091)* males do not mate very well.

lin-39 provides specificity to the Ras signaling pathway during vulval induction

Chapter three described interactions between the Hox gene *lin-39* and the Ras signaling cascade during induction of the *C. elegans* vulva. It is well established that activation of the Ras pathway is required for induction and patterning of the vulva, and it also has been known that this occurs within *lin-39's* domain of action. However, in *lin-39(-)* mutants, the cells that normally are competent to respond to Ras activation instead undergo an early fate transformation and fuse into a multinucleate syncytium, leaving them unable to respond. For this reason it had been difficult to assess whether or not *lin-39* is important at the time of vulval induction.

Using antibodies against LIN-39 I determined that *lin-39* is expressed in

the vulval cells at the time of induction, and that it is expressed in a pattern suggestive of regulation by Ras. I went on to show that *lin-39* expression is regulated by Ras, that both *lin-39* expression and Ras activation are necessary for vulval development to occur, and that *lin-39* provides regional specificity to the Ras signaling system such that vulval fates are adopted in response to Ras activation. This work demonstrated a new mechanism for fine-scale control of Hox gene expression, identified *lin-39* as a gene functioning downstream of the conserved Ras cassette, and provided insight into how activation of Ras leads to different outcomes in different tissues.

One confusing aspect of this work is the fact that while *lin-39* expression levels are controlled by the Ras signaling pathway, I was not able to demonstrate any importance for *lin-39* upregulation by Ras activation. Why might *lin-39* be upregulated in these cells? There are a number of possible reasons. I initially thought that graded levels of *lin-39* expression might be important for patterning the 1°, 2°, and 3° vulval fates, with high levels specifying the 1° fate and moderate levels the 2° fate. Multiple signaling systems specify 1° versus 2° fate: high levels of *lin-3/EGF* signal promotes the 1° fate, whereas lower levels promote the 2° fate; lateral signaling through *lin-12/Notch* is able to specify a 2° fate independent of Ras activation (Katz et al., 1995; Simske and Kim, 1995; reviewed by Kenyon, 1995). *lin-39* levels may be able to specify 1° versus 2° fates, but normally be overridden by these other signals. In *lin-12(-)* mutants all of the induced

VPCs adopts a 1° fate because there is no lateral signaling (Greenwald et al., 1983), and each expresses high levels of *lin-39* (data not shown)). It is possible that in these animals the high levels of *lin-39* are contributing to the 1° fate determination; therefore it would be interesting to examine the effects of different *lin-39* levels on VPC fate in *lin-12(-)* mutants. Interestingly, there is a great deal of variation in the way the vulval induction genes are used in different nematode species. For example, in some species there is a two-stage vulval induction, in others the gonad is not needed for induction, and in yet others there is only partial dependence on the gonad for induction (reviewed by Emmons, 1997; Sommer, 1997). It therefore seems quite possible that in some nematode species *lin-39* may be the critical determinant in patterning 1° versus 2° vulval fates. If so, the upregulation seen in *C. elegans* may reflect that evolutionary heritage.

Another potential role for *lin-39* upregulation was suggested by studying the effects of *hs-mab-5* on vulval development. In wild-type worms *hs-mab-5* has little effect on the vulval lineages (data not shown). Likewise, although the moderate reduction in *lin-39* activity caused by the *n709ts* mutation reduces vulval induction in some animals, it does not dramatically alter vulval fates (Fig. 3.5; (Ellis, 1985). However, I found that expression of *hs-mab-5* in *lin-39(n709ts)* animals caused the VPCs to undergo a dramatic homeotic transformation and adopt posterior, male-like PAG fates. This suggests that *lin-39* upregulation may act as a fidelity mechanism to prevent

interference by other Hox genes.

Our observation that replacement of *lin-39* by *mab-5* can cause the hermaphrodite VPCs to adopt fates characteristic of the male PAG may seem surprising. Because the vulva and PAG are sex-specific in wild-type worms it had been assumed that sex determining factors such as *tra-1* (Hodgkin, 1993)would play an important role in regulating their development. Of course these structures do develop in different A/P regions, so in hindsight it makes sense that the Hox genes are important in specifying which structure is made. What is fascinating about this example is that it illustrates how sexually dimorphic structures such as the gonad (males have no gonadal anchor cell) can interact with an A/P patterning system to induce a non-dimorphic tissue to adopt a sex specific fate.

I have proposed that inactivation of the negative regulator *lin-1* by Ras signaling causes multiple genes normally repressed by *lin-1* to become competent for expression, and that *lin-39* functions to selectively activate those genes specifically involved in vulval development. What might those genes be? After being induced, the VPCs divide multiple times, specifically reorient their division axes, and undergo dramatic morphological changes. Therefore it seems likely that *lin-39* will be found to control regulators of cell-division and cytoskeletal organization. Of course these are exactly the same genes that *mab-5* must control to produce the PAG. The interesting thing is that *lin-39* and *mab-5* somehow must also control the way in which these

regulators are deployed so that vulva or PAG fates are executed correctly. This is one of the most fascinating and relatively unexplored areas of developmental biology: what are the links between the assignment of developmental fate by transcription factors and the execution of specific morphogenic programs characteristic of the particular developmental fate.

The *C. elegans* vulva should be an excellent system in which to study the links between fate assignment and morphological development. In order to do so, the genes acting downstream of *lin-39* during vulval development need to be identified. One approach would be to use differential display or a *C. elegans* "gene chip" to identify transcripts differentially expressed in *lin-39(-)* and *lin-39(+)* worms at the time of vulval development. Sensitivity might be increased by doing this in a *lin-1(-)* background. Of particular interest would be messages coding for actin- and microtubule-associated proteins and other cytoskeleton components. The subcellular localization of these components could be examined to determine whether *lin-39* or *mab-5* modified their behavior. It would be best to combine such an approach with a genetic screen. Many of the components involved in vulval morphogenesis may also be involved in many other processes as well, so null mutations may be lethal. Nevertheless, it may be possible to find special alleles which have a vulval phenotype, but which are not lethal. Although a large scale screen for vulval mutants has been performed (Ferguson and Horvitz, 1985), the screen was biased towards finding mutations causing gross aberrations in vulval

induction. Both Eisenmann et al. (in preparation) and T. Herman have performed screens looking for mutants with a protruding vulva, in the hopes of finding downstream vulval genes. Pursuing the mutants identified in these screens, and perhaps repeating the screens to generate more mutants could be useful. Another approach would be to use a compound Nomarski microscope to screen staged animals during L3 lethargis to search for alterations in vulval lineages. A third interesting screen would be to look for suppressors of *lin-11*, a LIM domain transcription factor required for proper execution of 2° lineages (Freyd et al., 1990). *lin-11* mutations cause the 2° L L T N pattern of fates to be converted to L L L L; suppressors which restored the T and N fates might be gain-of-function mutations in genes required for those fates. Finally, (Rorth, 1996) has described a *Drosophila* screen in which a mobile enhancer/promoter is hopped throughout the genome, and then temporally and spatially limited expression of the Gal4 transactivator is used to achieve misexpression of targeted genes in tissues of interest. This approach allows identification of genes that might be missed due to lethality in more conventional screens. A similar screen done in *C. elegans*, with Gal4 expression driven by a vulval-specific promoter, could be a particular fruitful method of identifying downstream genes involved in the execution of vulval fates.

Lastly, I would like to note that *lin-39* may have a later, more direct role in vulval morphogenesis itself. Shortly after the first VPC division

lin-39 is downregulated in the 1° cell descendants and up-regulated in the outer 2° cell descendants (P5.pa and P7.pp, the cells that give rise to the L fates). After the next division it is expressed very strongly in the outermost L cells and then later very strongly in their descendants. Might *lin-39* have a role in distinguishing the L from the T and N fates, or in the final differentiation of the outer L cells and their descendants? This could be addressed by using the *lin-39(-); hs-lin-39* techniques described in chapter three. In this case, however, heat pulses would be directed to remove *lin-39* or to add ectopic *lin-39* after vulval development had already begun. Such an approach might reveal that *lin-39* is involved throughout the entire developmental course of the VPCs: first, to prevent their fusion; second, to specify vulval fates in response to Ras activation; and third, to help execute a particular subset of those fates. This would be an exciting way to further study the importance of fine-scale control of Hox gene expression.

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For reference

Not to be taken from the room.