INTERACTIONS OF MULTIPLE SIGNALING PATHWAYS GENERATE TAIL PATTERNING DURING THE DEVELOPMENT OF *CAENORHABDITIS ELEGANS*

Thesis by

Li (Lily) Jiang

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1999

(Submitted December 7, 1998)

© 1999

Li Jiang

All Rights Reserved

ACKNOWLEDGEMENTS

The birth of this thesis would not have happened without the encouragement and support of many people. I am very fortunate to have the opportunity to do my thesis research in Dr. Paul Sternberg's lab. Paul is a great scientist and a great mentor. His sincere love and dedication to science has made great influence on me. He is always genuinely enthusiastic about any progress I made. With his intuitions, insights and experiences, Paul helped me overcome many difficulties in my research. It is from his insightful vision that I started to taste the joy of research. As a mentor, he always sees the brightest side of his students and encourages us to move on. I am very grateful to Paul. I am proud of having him as my mentor. And I hope one day he will be proud of me as his student.

I did my first rotation in Dr. Howard Lipshitz's laboratory. It was when I did not know much about developmental and molecular genetics and my English proficiency was poor. Howard's easy going and truly caring character helped me ease in smoothly.

During the past five years, members of my thesis committee, Drs. Elliot Meyerowitz, Mel Simon, Scott Fraser, and Kai Zinn, have always been encouraging and supportive. What they have taught me is not only knowledge but more importantly how to be a real scientist. Looking from a distance, I can't help admiring their sharp and deep thinking, their dedication to science, their commitment to education, as well as their ability to balance career and life. They will always be my models to look up to.

I am blessed to have a sincere friend, Maureen Barr. Whenever I need somebody to share my joy, my pain, or my trouble, Maureen is always there listening to me, cheering for me, or soothing my heart, helping me deal with my problems, no matter research or life-wise. Jing Liu, Minqin Wang and I are like sisters. We shared a lot of precious memories together. Life would be more difficult and less joyful without them. During the last few months, Minqin and I had quite a lot of discussions on future plans for life and career. I enjoyed it.

My thanks extend to many friends who happen to cross my life and have touched my heart in many, many ways, Sunping Zheng, Fan Wang, Peter Becker, Jian Hua, Tom Clandinin, Helen Chamberlin, Michele Lamka, Nancy Winfree, Keith Brown, Bino Palmer, and Nadeem Moghal. Thanks to every member in the Sternberg lab, previous and current, for making the lab a fun place.

Most importantly, I would thank my family for everything they have done for me. Allan and I met here at Caltech. We got married here. We started a new life here. I cannot imagine what life would be without him. We are not only life partners but also real soul mates. There were up and downs in our road. But whenever I need support, I need inspiration, or I need a critical eye, he is always there for me. Although we are in totally different fields of research, we always do practice talk with each other and revise each other's manuscript. We often discuss the philosophy of research, the meaning of life, and the balance in between. Our sharing of life has made both of us better and stronger persons. Justin is a miracle. He has brought so much joy

and so much new meaning to our lives. It is after his birth that I started to fully appreciate and treasure the meaning of life.

Being a parent myself makes me realize how lucky I am to have wonderful parents like my mom, Baogui Zhao and my dad, Guansheng Jiang. Raising me right and giving me the best education is the most important thing in their lives. They sacrificed a lot for that. Ever since I was able to make decisions on my own, they have always trusted my choice, supported me, and encouraged me to fulfill my dreams. It is the perseverance I inherited from them that made me succeed. Their love and support are invaluable to me and are always there for me. I am especially grateful to my parents and my sister for taking care of my Justin during the past few months.

ABSTRACTS

The specification of a certain cell fate and cell function involves three fundamental questions. First, what genes control the specification of a certain cell fate? Second, how do multiple extrinsic and intrinsic factors interact to coordinate this developmental decision? Third, what genes regulate cellular behaviors and the exertion of cell functions? I addressed these developmental issues by studying the patterning of *C. elegans* tail.

In hermaphrodite tail, the specification of the P12 neuroectoblast fate involves three classes of genes. The conserved EGF signaling pathway is utilized for P12 fate specification, and the LIN-3 epidermal growth factor is an inductive signal to promote the P12 fate. Hox gene *egl-5* is a downstream target of the *lin-3/let-23* pathway in specifying P12 fate. Wnt pathway components *lin-44* and *lin-17* act synergistically with the EGF pathway in the specification of the P12 fate. The Wnt pathway may function early in development to regulate the competence of the cells to respond to the LIN-3 inductive signal.

A SPD neuron specific marker is used to study the terminal cell fate specification during *C. elegans* male spicule development. The specification of glial vs. neuronal cell fate in a canonical neurogenic lineage is rather dependent on Wnt signaling. Inactivation of the *lin-17*-mediated Wnt signaling pathway transforms the SPD sheath cell into its sister, the SPD neuron. A genetic screen identified a new mutant, *son-1*, which displays the same cell fate transformation. *son-1* genetically interacts with *lin-17* in several developmental processes. Cloning of *son-1* revealed that it encodes an

HMG1/2-like DNA binding protein. This result provides evidence for the specific in vivo functions of the HMG1/2-like proteins. SON-1 acts in the Wnt responding cells to facilitate gene transcription.

During spicule morphogenesis, the spicule neurons and sheath cells are dispensable while the socket cells are essential for spicule morphology. The two functional aspects of socket cells, spicule elongation and secretion of spicule cuticle, are genetically separable and are controlled by the FGF and TGF-β signaling pathways and a gene defined by the *sy170* locus, respectively.

TABLE OF CONTENTS

Acknowledgements	iii
Abstract	vi
Chapter I: Information integration in the specification of cell fate	I-1
Information integration in the specification of cell fate	I-2
C. elegans tail patterning	I-12
Thesis overview	I-19
References	I-21
Figure 1. Development of the ventral cord precursor cells and tail blast cells in *C. elegans* hermaphrodites and males.	I-28
Figure 2. Multiple signaling pathways mediate several developmental processes in *C. elegans*.	I-30
Figure 3. Early stage B lineage development.	I-32
Chapter II: Interactions of EGF, Wnt and HOM-C genes specify the P12 neuroectoblast fate in *C. elegans*	II-1
Chapter III: A screen for mutants defective in neurogenesis during *C. elegans* male spicule development	III-1
Abstract	III-2
Introduction	III-3
Materials and Methods	III-5
Results	III-8
Discussion	III-23

References III-29

Figure 1. The B lineage. III-33

Figure 2. *gpa-1::lacZ* is expressed in SPD neurons specifically. III-36

Figure 3. *gpa-1::lacZ* expression in cell(s) ablated animals. III-38

Figure 4. *gpa-1::lacZ* expression in various mutants examined. III-40

Figure 5. Screen scheme. III-42

Figure 6. Mutants recovered from the F1 clonal screen. III-43

Table 1. *gpa-1::lacZ* is expressed in SPD neurons specifically. III-46

Table 2. F, U, Y.p ablation. III-47

Appendix: Other molecular markers that are expressed in the spicule neurons III-48

Chapter IV: An HMG1-like protein facilitates Wnt signaling in *C. elegans* IV-1

Summary IV-2

Introduction IV-3

Results IV-6

Discussion IV-19

Materials and Methods IV-26

References IV-32

Figure 1. The B lineage. IV-38

Figure 2. SPD neuron marker is expressed in 2-cell per spicule in Wnt mutants, *lin-44* and *lin-17*, and *son-1(sy549)*. IV-40

Figure 3. Mapping and cloning of *son-1*. IV-43

Figure 4. Sequence of the *son-1* gene. IV-45

Figure 5. *son-1::GFP* is expressed in all somatic nuclei. IV-48

Figure 6. Disruption of *son-1* function by *RNAi* and overexpression of POP-1 result in the same spectrum of phenotypes. IV-50

Figure 7. Structure/function analysis of HMG boxes. IV-52

Figure 8. Model for SON-1 function. IV-54

Table 1. Both *lin-17(n698)* and *son-1(sy549)* confer a SPD sheath to neuron fate transformation. IV-56

Table 2. *son-1(sy549)* enhances *lin-17(n698)* phenotypes to *lin-17*-null like. IV-57

Chapter V: Socket cells mediate spicule morphogenesis in *C. elegans* males tail V-1

Abstract V-2

Introduction V-3

Materials and Methods V-6

Results V-7

Discussion V-14

References V-19

Figure 1. The composition of *C. elegans* male spicule and the spicule lineage. V-21

Figure 2. The socket cells and the B.paa cell are responsible for spicule morphology and gubernaculum morphology respectively. V-24

Figure 3. *egl-17::GFP* expression pattern in the male tail. V-26

Figure 4. Phenotypes and marker expression patterns in *lin-48(sy548)* and *sy170* mutants. V-28

Figure 5. *egl-17::GFP* expression in *sma* mutants. V-30

Figure 6. Developmental pathway of spicule morphogenesis. V-32

Chapter VI: Summary VI-1

Chapter I

Information integration in the specification of cell fate

INFORMATION INTEGRATION IN THE SPECIFICATION OF CELL FATE

During the development of a multicellular organism, cells are specified to adopt certain fates in a fixed stepwise fashion. Cell fate specification involves information integration of both environmental conditions (extrinsic factors) and the state of cell competence (intrinsic factors). In a certain developmental field, a cell receives signals from its neighboring cells both in the same field and outside. The temporal and spatial distribution of these signaling molecules can generate a wide variety of cell fates. Exactly what fate a cell will assume depends on the ability of the cell to sense the signals and to respond accordingly. This process presents many fundamental questions for developmental biologists. What are these signaling molecules? What are the temporal and spatial distributions of these signals? What kinds of interactions exist between multiple signaling pathways? How is cell competence established? What is the time window of cell competence? How does a cell integrate information from a multitude of extrinsic and intrinsic factors?

In this review I will attempt to address these questions from the cell's perspective taking into account external forces (the environment), intrinsic cellular properties, information processing, and decision execution. I will first discuss the parameters essential for cell fate specification and then discuss the underlying biological lessons.

The signals ----- information a cell receives

In order to make a choice of cell fate, a cell is usually exposed to a certain environment and receives multiple information. This information is encoded by signaling molecules released to its environment. Different signaling molecules act through distinct cellular pathways and induce specific cell fates. For example, during *Drosophila* embryogenesis, a stripe of two rows of Engrailed (En) expressing cells differentiate into two distinct cell fates, anterior smooth cuticle fate and posterior denticle cuticle fate, in a signal-dependent manner. Signaling of *wingless (wg)*, a member of the Wnt family gene, directs the anterior row of En cells to adopt anterior smooth cuticle fate (Cabrera et al., 1987; Rijsewijk et al., 1987; Baker, 1987; Dougan and DiNardo, 1992), while *spitz*, which encodes a ligand for the *Drosophila* epidermal growth factor receptor (DER), specifies the posterior denticle cuticle fate (Rutledge et al., 1992; Schweitzer et al., 1995a; O'Keefe et al., 1997). For certain signaling molecules, signal concentration and duration may provide additional instructions for receiving cells. Some signaling molecules act at several concentration thresholds, with each threshold potentially eliciting a distinct response. Therefore, the quantitative difference of a signaling molecule can result in a qualitatively different outcome. Examples include signaling molecules that act as morphogens (e.g., HEDGEHOG, Heemskerk and DiNardo, 1994; LIN-3, Katz et al., 1995). Other signaling molecules may not be diffusable, but rather require cell-cell contact to transduce their activities (e.g., BOSS ligand and its receptor SEVENLESS, Reinke and Zipursky, 1988). The same signaling molecule may

also possess combined modes of action via two forms of protein processing, a soluble form that acts in the range of several cell-diameters, and a membrane-bound form that requires cell-cell contact for its function. The two forms of proteins may be used for precise patterning, or they may trigger different receptor activities and regulate different cellular behaviors (e.g., KIT ligand, Huang et al., 1992; Miyazawa et al., 1995; Wehrle-Haller and Weston, 1995).

During most developmental processes, a cell receives multiple signaling inputs. These signals may act sequentially or simultaneously on the receiving cell. They may function independently or interactively. Interactions can be cooperative or antagonistic. The relative strength of each signal may also contribute to the outcome. Interactions between multiple signals may generate new properties that each individual signal does not have, thereby adding complexity for the receiving cell to interpret and diversity for the outcome. For example, the leg imaginal discs of *Drosophila* are subdivided into three compartments. Localized expression *of hedgehog (hh), decapentaplegic (dpp),* and *wg* mark the boundaries along the posterior, dorsal anterior, and ventral anterior compartments, respectively. *hh* directs the expression of *dpp* and *wg* and sets up the anterior-posterior axis (Basler and Struhl, 1994). *wg* alone specifies the ventral fate and organizes the dorsal-ventral axis (Couso et al., 1993; Struhl and Basler, 1993). Together with *dpp*, *wg* induces the expressions of two homeodomain proteins, Aristaless and Distalless, and establish the proximal-distal axis of leg imaginal discs (Campbell et al., 1993; Diaz-Benjumea et al., 1994). Thus, the

concerted action of two signaling molecules, WG and DPP, creates a unique outcome.

The state of competence ----- properties a cell possesses

How a cell responds to environmental signals depends on its properties. Each cell has its own developmental history. A cell can remember its history and change its properties accordingly so that it may respond to the same signal differently at different developmental times. There are several ways a cell may change its properties. First, asymmetric localization of intrinsic factors during cell divisions may produce two different daughter cells. The inherited intrinsic factors may influence cell responsiveness to certain signals and bias toward certain developmental potentials. For example, the Numb protein is asymmetrically localized in a dividing precursor cell and is preferentially segregated into one of the two daughter cells in the *Drosophila* sensory organ precursor (SOP) lineage (Rhyu et al., 1994). Numb appears to inhibit Notch activity, which mediates the interaction between the two daughter cells (Guo et al., 1996). Thus, the asymmetric inheritance of Numb makes the cell-cell interaction become asymmetric. Second, since cell fate specification is a multi-step process, a cell may be primed for a particular fate through pre-patterning. Conserved hox genes are usually expressed early in embryogenesis to lay down the general body plan along the anterior-posterior axis. Localized expression of different hox genes can prepare cells to respond to local signals. In the vulval development of *C. elegans*, hox genes, *lin-39* and *mab-5*, set up the sensitivity of the vulval precursor cells to the inductive signal LIN-3/EGF (Clandinin et

al., 1997). Third, cell competence can also be coupled with cell cycle. An internal clock has been postulated to measure the number of cell divisions and trigger the change of cell properties at fixed times (Reh and Cagan, 1994; e.g., laminar fate specification of cortical neurons, McConnell and Kaznowski, 1991). Fourth, a cell may adjust its own properties based on the differentiation state of its neighbors. The Notch/Delta pathway is widely used to mediate cell-cell interaction between neighboring cells (reviewed in Greenwald, 1998). A cell can sense its neighbors by comparing the relative activity of the Notch/Delta pathway and choose a fate distinct from its neighbors.

A change in cell properties may change the cell's response to certain signals during development. The major signaling pathways are usually used at multiple times and at different places throughout animal development. However, they may have different functions and induce different cell fates depending on the intrinsic properties of the responding cell. A good example is the repeated use of the *Drosophila* DER pathway in the development of *Drosophila* eye (Freeman, 1996). Successive rounds of DER activation recruits all types of ommatidial fates to the developing ommatidium. Which fate DER triggers depends on the developmental stage of the receiving cell. During the larval stage, activation of the DER pathway causes cells close to the eye morphogenetic furrow to become outer photoreceptors, and the cells in more mature ommatidia to assume R7 or cone cell fates. If the DER pathway is activated at the pupal stage, it will trigger pigment cell fate. It is very likely that as the cells age, certain intrinsic properties change, resulting in unique responses to the same signaling molecule.

Information integration ----- how a cell makes decisions

Signal transduction is the process by which a cell integrates individual signals and executes fate decision. During the past two decades, several major signal transduction cascades have been discovered and characterized with pathway components being identified and ordered (e.g., the RAS, Wnt, and TGF-β pathways, reviewed in Sternberg and Alberola-Ila, 1998; Cadigan and Nusse, 1997; Whitman, 1998). A common paradigm of signal transduction has emerged. An extracellular signal binds to its receptor at the surface of the receiving cell and stimulates receptor activity. Activated receptor transduces this activity through a cascade of cytoplasmic kinases to the nuclear transcription factors. Modifications of transcription factors, such as phosphorylation/ dephosphorylation or subcellular localization, may activate or repress target gene expression hence implementing the message conveyed by the signal. These coordinated changes lead to the specification of certain cell fates. Multiple levels of regulation exist for each signaling pathway. Positive and negative regulators as well as positive and negative feedback loops act at each level of signal transduction: receptor activation, kinase activity, and transcription factor activity.

A cell must orchestrate multiple environmental signals along with its intrinsic properties into a proper developmental response. Integration of signaling information can happen at the cell surface, in the cytoplasm, in cell nucleus, or at multiple points. One example for signal integration at the cell surface involves the regulation of DER activation by Sptiz and Argos. Both Spitz and Argos are secreted proteins that contain a single EGF motif

(Rutledge et al., 1992; Freeman et al., 1992). Spitz is a ligand that activates DER activity, while Argos inhibits DER activation (Schweitzer et al., 1995a, 1995b). The balance between the two signals regulates the state of activation of the DER pathway and determines cell fate. Moreover, transcription of *argos* has been shown to be induced by DER activation, establishing an inhibitory feedback loop (Golemo et al., 1996). These complex interactions ensure that a gradient of pathway activation be created and a pattern of cell fates be generated.

In many cases, information integration occurs in the cytoplasm. Generally, the greater the number of components present in a signaling pathway, the greater the potential for cross-talk and signal integration. Many signaling pathways rely on a protein kinase cascade to transduce signals from the cell surface to the nucleus. Such protein kinases are often positively or negatively regulated by serine/threonine or tyrosine phosphorylation or both, making them prime targets for cross-talk between signaling pathways. Each protein kinase can have multiple phosphorylation sites. Multiple signaling pathways can converge on the same kinase by regulating the phosphorylation/dephosphorylation status of different sites and thereby regulating kinase activity. For example, the mitogen-activated protein kinase (MAPK) pathway is a highly conserved signaling module that is extensively used for signal transduction. MAPK can be activated by epidermal growth factor (EGF) and other agents through sequential activation of the guanine nucleotide exchange factor SOS, the guanine nucleotide binding protein RAS, and the protein kinases RAF, MAPK kinase, and MAPK. In NIH 3T3 fibroblasts, activation of the MAPK pathway by EGF is inhibited by

activation of the cAMP pathway at the level of RAF activation (Cook and McCormick, 1993; Wu et al., 1993). This inhibition is proposed to be a result of direct inhibitory phosphorylation of RAF by protein kinase A (PKA), a component of the cAMP signaling pathway. PKA-dependent phosphorylation of RAF inhibits RAF catalytic activity and RAF binding to RAS (Hafner et al., 1994). In the case of TGF-β pathway, multiple SMAD protein complexes transduce the signal activity from the TGF-β receptor to downstream transcription factors (reviewed in Whitman, 1998). The MAPK signaling pathway has the capacity to antagonize or synergize with the TGF-β signaling depending on the developmental context. The signals appear to be integrated at the level of SMAD protein phosphorylation. Depending on which SMAD protein is phosphorylated by MAPK, the effect could be inhibitory or stimulatory. Cross-talk between signaling pathways is essential for a cell to properly evaluate its environmental milieu and to coordinate necessary physiological changes.

Convergence of signaling cascades also often occurs in the nucleus at the level of gene transcription. If a gene regulatory region contains multiple signal-regulated activation elements, only stimuli that can simultaneously activate all of these elements may activate gene transcription, while a stimulus that activates only a subset will not. Such is the case for regulation of the *Drosophila* homeotic gene *Ultrabithorax (Ubx)* expression by *wg* and *dpp* in the visceral mesoderm (Thuringer and Bienz, 1993). Both a *wg*-response element (WRS) and a *dpp*-response element (DRS) have been identified in an enhancer region of *Ubx* (Riese et al., 1997). A nuclear factor

LEF-1, the most downstream known component of the conserved *wg* signaling pathway, has been shown to bind the WRS in the *Ubx* enhancer and activate gene expression. Moreover, the two signaling pathways, *wg* and *dpp*, appear to regulate *Ubx* gene expression synergistically (Riese et al., 1997). Combination of this kind potentially generates unique effects that neither pathway could evoke in isolation. For example, activation of *wg* and *dpp* signaling pathways direct *aristaless* and *distalless* expression in the *Drosophila* leg imaginal discs, generating the proximal-distal axis of the legs (Campbell et al., 1993; Diaz-Benjumea et al., 1994). While it is possible that the two signaling pathways converge at the level of gene transcription, the WRS and DRS remain to be identified in the promoter/enhancer of *aristaless* and *distalless*.

Once a cell fate decision is made, execution of cell fate relies on coordinated responses for all the aspects of cell behaviors, proliferation, gene expression, morphological change, migration, etc.. The post cell fate specification regulation of cellular behaviors may also be mediated by multiple intrinsic and extrinsic factors.

The logic of cell fate specification

Several basic parameters required for cell fate decisions have been discussed. The first parameter, the signal, is a function of three variables, the mode of action of the signal, the spatial distribution of the signal, and the temporal distribution of the signal. The second parameter is the competence of the responding cell, which may change throughout development. The third parameter considers the potential interactions between multiple extrinsic

and intrinsic factors. Biologists have identified several key signaling pathways that play important roles in cell fate specification through development. Most of those signaling pathways are evolutionarily conserved through metazoa. Many strategies used in nature for signaling pathway regulation, information integration and cell fate determination have been discovered. Yet what we found is the loss of simplicity and the increase of complexity. Overlapping and partially redundant systems are often employed to ensure cell fate specification. A certain molecule may possess dual functions with opposite roles depending on the developmental context. Two signaling pathways can interact at multiple levels and may be integrated differently in different tissues or organisms. Coupling of the same two signaling pathways may also have opposite effects depending on developmental context. From an evolutionary point of view, this is not difficult to understand. What are conserved through evolution are the basic modules of the signaling pathways and regulatory loops, and the basic strategies for making developmental decisions. When changes are needed, the most efficient way is to modify pre-existing components. Thus the combination of these modules and strategies may not be the simplest or most efficient. Rather subtle changes of certain parameters may have profound and complex effects on information integration. Hence is the complexity and diversity of the world. As Cox and Peifer (1998) pointed out "Nature is a home handywoman. Constrained by evolution, she does the job with the tools at hand, using a screwdriver for a hammer if necessary."

Perspectives

Traditional genetic screens (phenotype based) have been powerful tools in identifying key players involved in certain developmental process and in ordering signaling pathway components. From qualitative phenotypic analysis, what we see is only the overall outcome of a cell's final decision. In vitro biochemical studies can help dissect the process of signal transduction and pathway interactions. However, physiological relevance must be demonstrated in vivo. Considering the complexity of information integration, the existence of positive and negative feedback loops and mutual regulation, we need to design better screen strategies and develop quantitative phenotypic analyses in order to decipher the process of information integration in vivo. Sensitized or conditional alleles have been useful in screens for new components or regulators of certain signaling pathways. Certain reagents have been developed to allow detection of the activation/inactivation state of certain signaling molecules in vivo. Many cell fate specific markers have been identified and can be used to study the process of cell fate specification in detail. Eventually, these tools and approaches will aid in the discovery of the smart strategies nature has evolved and better understanding of the logic of nature, the activity of each signaling pathway component will be monitored and the relationship between them will be quantitatively assigned.

C. ELEGANS TAIL PATTERNING

Caenorhabditis elegans is an excellent model organism to genetically and molecularly dissect interactions between multiple developmental

pathways. I have been focusing on the development of the *C. elegans* tail. In the *C. elegans* tail region, there are several blast cells at hatching, P9, P10, P11, P12, F, U, Y, K, B, and T cells (Figure 1). Postembryonic divisions of these cells generate patterning of the tail region (Sulston and Horvitz, 1977). Sexual dimorphism in cell division patterns and organ generation exists. In the male, P10.p and P11.p cells generate the hook, the B blast cell generates male spicules, the Y cell generates cells of the post cloaca sensillium, and the T cell gives rise to a subset of the rays. These are all copulatory structures that are required for mating behavior. F and U cells form part of the proctodeum and mediate the connection of the gonad to male mating structures. In the hermaphrodite, F, U, Y, and B cells do not divide, forming part of the rectum. P(9-11).p cells take on a hypodermal fate and fuse with hyp7. P12.p generates a unique hypodermal fate, hyp12. The K cell is an essential rectal epithelial cell in both hermaphrodite and male. Ablation of K cell at hatching results in growth arrest and death at L1 stage. Previous studies have shown that cell fate specification in the tail region requires several types of regulation involving cell signaling and interactions. More interestingly, several signaling pathways are found to be utilized in both males and hermaphrodites to generate the tail patterning.

P(9-12).p development

P1 to P12 are six pairs of ventral cord precursor cells that are disposed bilaterally at hatching. Several hours after hatching these cells start to migrate into the ventral cord through cytoplasmic extension to form a consecutive row of twelve ventral cord cells, numbered P1 to P12 from

anterior to posterior (Figure 1). The anterior daughters of these cells, Pn.a, are neuroblasts and generate the ventral cord neurons. The posterior daughters, Pn.p, give rise to epidermal fates. In hermaphrodites, P(3-8).p are vulva precursor cells (VPC). The VPCs divide in a fixed pattern and form a functional vulva that is required for egg-laying. The patterning of vulva is centered on P6.p being primary vulval fate, P5.p and P7.p being secondary fate, and P3.p, P4.p, and P8.p being tertiary fate. This pattern is organized by the gonadal anchor cell (AC) which resides above the P6.p cell (Figure 2A). Ablation of the AC abolishes vulval induction and patterning. P(1-2).p and P(9-11).p do not divide but rather fuse with the large epidermal syncytium hyp7. P12.p divides once to generate P12.pa, which becomes a unique epidermal cell, hyp12, and P12.pp, which undergoes cell death. Although the functions of P11 and P12 are not clear, they are both competent to express P12 fate at hatching. By cell ablation experiments, Sulston and White (1980) demonstrated that prior to migration both P11 and P12 cells are able to express the P12 fate; and if only a single cell is present, it will adopt the P12 fate. Therefore P12 represents a primary fate, while P11 is a secondary fate (Figure 2B).

In males, Pn.a cells are also responsible for generation of the ventral cord neurons. All P(1-8).p cells do not divide and fuse with hyp7. P(9-11).p undergo extra rounds of cell division to generate the hook structure which is required for vulva location behavior during male mating (Sulston and White, 1980; Liu and Sternberg, 1995). Patterning of the hook is oriented from posterior to anterior with P11.p being primary fate, P10.p being secondary fate, and P9.p being tertiary fate (Sulston and White, 1980; Figure 2C).

Ablation of P11.p results in the replacement of primary fate by P10.p and secondary fate by P9.p, and an ectopically located hook can be formed. Ablation of both P11.p and P10.p can even make P9.p become primary fate. The fate replacement is always from anterior to posterior. In the differentiation of the most posterior hypodermal cell P12.p, similar regulation exists in males as it is in hermaphrodites. P11 can replace P12 provided that ablation of P12 is carried out prior to nuclear migration (Sulston and White, 1980).

For the patterning of P(9-12).p in both males and hermaphrodites, a tail "organizer" might exist posterior to the P12.p cell to regulate the patterning of the posterior ventral cord precursor cells. This tail "organizer" plays a similar role as the AC does for VPC patterning. Interestingly, the sets of genes that regulate vulva patterning are found to control the tail patterning. In the formation of the vulva (reviewed in Sundaram and Han, 1996; Kornfeld, 1997), the AC signal is encoded by *lin-3*, a *C. elegans* homologue of the epidermal growth factor (EGF) family gene. LIN-3 transduces its activity through a tyrosine kinase receptor, encoded by *let-23*, to activate downstream RAS signaling. Activation of the *lin-3/let-23* signaling pathway induces vulval fates. Interaction between primary and secondary vulval fates mediated by a Notch homologue, *lin-12*, is required for the expression of the secondary vulval fate. Vulval fate induction and VPC competence to the inductive signal LIN-3 are also regulated by a hox gene *lin-39*, which is expressed in the middle region of the ventral cord (Clandinin et al., 1997; Maloof and Kenyon, 1998). The patterning of the tail also requires the activities of the *lin-3/let-23* EGF signaling pathway, the Notch

homologue *lin-12*, and hox genes *mab-5* and *egl-5*. Inactivation of the *lin-3/let-23* EGF signaling pathway results in P12 to P11 fate transformation in both males and hermaphrodites (Fixsen et al., 1985; Aroian et al., 1991; Han et al., 1990; Chapter II). Loss-of-function mutants of *egl-5* display this same phenotype, while loss-of-function mutants of *mab-5* display reciprocal transformations of either P11 to P12 or P12 to P11 (Kenyon, 1986; Chisholm, 1991). *lin-12* is found to mediate the interactions between the hook group P(9-11).p cells in males (Greenwald et al., 1983). As in vulval induction, gain-of-function *lin-12* promotes secondary fate and results in extra hook(s) formed ectopically; loss-of-function *lin-12* leads to loss of secondary fate and loss of hook formation. In addition, mutations in the *lin-17* gene, which encodes a *Drosophila* FRIZZLED-like seven-transmembrane protein and is a putative receptor for Wnt signaling, also result in lineage abnormalities in P7.p during vulva development and in P10.p and P11.p during hook formation (Sternberg and Horvitz, 1988; Sawa et al., 1996). It is not yet clear what role the Wnt signaling pathway plays in these developmental processes.

By analogy to vulval patterning, tail patterning is an interesting model system in which to dissect the interactions between the EGF, Wnt, and Notch signaling pathways and the Hox genes. Moreover, it will be interesting to determine whether similar strategies are employed for information integration in these developmental processes.

Development of male specific blast cells

F, U, Y, and B cells are male specific blast cells. Generation of these blast cells has not been extensively studied until recent years. Hox genes

mab-5 and *egl-5* play a role in the specification of these blast cell fates. Loss-of-function mutation in *mab*-5 results in abnormal F, U, and B lineages with the B lineage mimicking the Y lineage. Loss-of-function mutation in *egl-5* affects F, U, and B lineages. A genetic screen based on abnormal male spicule morphology has yielded several mutations that disrupt the male specific blast cell fates (Chamberlin, 1994; Chamberlin et al., 1997). Several loci defined by those mutants have been cloned, and so far they all encode putative transcription factors (Chamberlin et al., 1997; H. Chamberlin, personal communication).

Of all these male specific blast cells, B lineage development is most complex. The B cell is the progenitor of the male spicule structure. Multiple signaling pathways and complex cell-cell interactions are used through different steps of B lineage development to control cell fate specification (Figure 3). The first division of the B cell is asymmetric, generating a larger anterior-dorsal daughter B.a and a smaller posterior-ventral daughter B.p. The asymmetry of this division is controlled by the genes *lin-44* (Herman and Horvitz, 1994), *lin-17* (Sternberg and Horvitz, 1988), and *vab-3* (Chamberlin and Sternberg, 1995). Epistatic analyses suggest that LIN-44 acts via LIN-17 to promote the posterior B.p fate (Chamberlin and Sternberg, 1995). The Pax-6 class transcription factor VAB-3 is required for the B.a fate and acts downstream of *lin-17* (Chisholm and Horvitz, 1995; Chamberlin and Sternberg, 1995). B.a generates all the cells of the spicules. By the end of L2 stage, B.a has divided three times to form a ring of eight cells. B.p divides once generating two cells (B lineage 10-cell stage). A network of cell-cell interactions occurs and leads to the specification of six distinct precursor cell

types at 10-cell stage, designated as α, β, γ, δ, ε (2), and ζ (2). F, U, and Y cells provide positional cues for the specification of cell fates at the 10-cell stage. A signal mediated by the F and U cells is required to promote the anterior fates (α, γ, ε) (Chamberlin and Sternberg, 1993). Ablation of F and U cells results in an anterior to posterior fate transformation. This signaling event is likely to be controlled by the *lin-3/let-23* EGF signaling pathway (Chamberlin and Sternberg, 1994). First, the B lineage defects caused by loss of function mutations in genes of the *lin-3/let-23* pathway, i.e., *lin-3, let-23, sem-5, let-60*, and *lin-45*, resemble those seen in F and U ablated males. Second, overexpression of LIN-3-EGF can compensate for the lack of F and U cells. The posterior fates (β, δ, ζ) are promoted by a signal from the Y.p cell and its progeny. When the Y.p cell is ablated, some of the posterior cell lineages are abnormal. *lin-12*, the *C. elegans* Notch homologue, mediates the interaction between γ and δ fates, which have been previously shown to be developmentally equivalent (Sulston and White, 1980; Chamberlin and Sternberg, 1994).

At the early L3 stage, the cells undergo short range migration to assume their correct positions. During L3 stage, these 10 cells continue to divide to generate spicule neurons, sheath cells, socket cells, and connective tissues. During mid-L4 to L4 lethargus, the male tail undergoes extensive reshaping and morphological changes with spicule morphogenesis occurring. The cells in the male tail region move anterodorsally to form correct connections. A pair of elongated spicules is formed with a layer of sclerotized refractive spicule cuticle laid down outside each spicule and retractor and

protractor muscles connect to each spicule. Each of the terminally differentiated B.a progeny contributes to spicule morphogenesis and spicule function. Different spicule neurons mediate specific steps of male mating behavior (Liu and Sternberg, 1995). For spicule morphogenesis, it has been suggested that the muscle attachment to the proctodial cells plays an essential role in spicule elongation. Ablation of muscle precursor cell results in failure of spicule morphogenesis therefore crumpled spicules (Sulston et al., 1980). Inferring from electron micrographs, one of the ε progeny appears to form a special contact with spicule retractors at the initial stage of spicule elongation.

Late stage B lineage cell fate specification and spicule morphogenesis are complex and interesting developmental processes. Yet due to the complexity of the lineage and the difficulty in assigning cell fate based on nuclear morphology and position, it has been difficult to study these processes. Study of terminal cell fate specification and morphogenesis relies on cell fate specific molecular markers. The rapid development molecular biology techniques coupled with the *C. elegans* Genome Project provides an opportunity to identify a variety of cell fate specific molecular markers. Proper usage of these markers can greatly facilitate the study of developmental processes.

THESIS OVERVIEW

In my thesis research I have concentrated on the process of cell fate specification and explored this problem from three different angles. In Chapter II, I describe how multiple extrinsic and intrinsic factors act together

to specify the P12 fate. Cell fate determination and information integration strategies, such as parallel action of two signaling pathways, Wnt and EGF, and regulation of hox gene expression by the EGF signaling pathway, are discussed. In Chapter III, I describe the identification of a spicule neuron fate specific marker and the study of spicule neurogenesis based on this marker. A genetic screen looking for mutants that specifically affect marker expression in the spicule region is described, and several mutants are identified and analyzed. In Chapter IV, I describe the involvement of the Wnt signaling pathway in the neuronal vs. non-neuronal fate decision during male spicule development. The genetic characterization and molecular cloning of one mutant recovered from the screen described in Chapter III are studied. It defines an HMG1/2-like DNA binding protein. Its possible role in Wnt signaling is addressed in detail. In Chapter V, I describe the functions of spicule socket cells in spicule morphogenesis. A molecular marker for socket cell fate is identified. The developmental pathway for spicule morphogenesis is further dissected. Chapter VI summarizes my thesis research and discusses the biological implications of my findings.

REFERENCES:

Aroian, R. V., and Sternberg, P. W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. Genetics 128: 251-277.

Baker, N. E. (1987). Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila* - the spatial-distribution of a transcript in embryos. EMBO-J 6: 1765-1773.

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by HEDGEHOG protein. Nature 368: 208-214.

Cabrera, C. V., Alonso, M. C., Johnston, P., Phillips, R. G., Lawrence, P. A. (1987). Phenocopies induced with antisense RNA identify the *wingless* gene. Cell 50: 659-663.

Cadigan, K. M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Gene & Dev. 11: 3286-3305.

Campbell, G., Weaver, T., and Tomlinson, A. (1993). Axis specification in the developing Drosophila appendage: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. Cell 74: 1113-1123.

Chamberlin, H. M., and Sternberg, P. W. (1993). Multiple cell interactions are required for fate specification during male spicule development in *Caenorhaebditis elegans*. Development 118: 297-324.

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

Chamberlin, H. M., and Sternberg, P. W. (1995). Mutations in the *Caenorhabditis elegans* gene *vab-3* reveal distinct roles in fate specification

and unequal cytokinesis in an asymmetric cell-division. Devel. Biol. 170: 679-689.

Chamberlin, H. M., Palmer, R. E., Newman, A. P., Sternberg, P. W., Baillie, D. L., and Thomas, J. H. (1997). The PAX gene *egl-38* mediates developmental patterning in *Caenorhabditis elegans*. Development 124: 3919-3928.

Chisholm, A. (1991). Control of cell fate in the tail region of *C. elegans* by the gene *egl-5*. Gene & Dev. 111: 921-932.

Chisholm, A. D., and Horvitz, H. R. (1995). Patterning of the *Caenorhabditis elegans* head region by the PAX-6 family member *vab-3*. Nature 377: 52-55.

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

Cook, S. J., and McCormick, F. (1993). Inhibition by cAMP of RAS-dependent activation of Raf. Science 262: 1069-1072.

Couso, J. P., Bate, M., and Martinez-Arias, A. (1993). A *wingless*-dependent polar coordinate system in *Drosophila* imaginal discs. Science 259: 484-489.

Cox, R. T., and Peifer, M. (1998). *Wingless* signaling: the inconvenient complexities of life. Curr. Biol. 8: 140-144.

Diaz-Benjumea, F. J., Cohen, B., and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature 372: 175-179.

Dougan, S., and Dinardo, S. (1992). *Drosophila wingless* generates cell type diversity among *engrailed* expressing cells. Nature 360: 347-350.

Fixsen, W., Sternberg, P. W., Ellis, H., and Horvitz, H. R. (1985). Genes that affect cell fates during the development of *Caenorhabditis elegans*. Cold Spring Harbor Symp. Quant. Biol. 50: 99-104.

Freeman, M., Klambt, C., Goodman, C. S., and Rubin, G. M. (1992). The *argos* gene encodes a diffusable factor that regulates cell fate decisions in the *Drosophila* eye. Cell 69: 963-975.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell 87: 651-660.

Golemo, M., Schweitzer, R., Freeman, M., and Shilo, B. Z. (1996). *Argos* transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. Development 122: 223-230.

Greenwald, I. S., Sternberg, P. W., and Horvitz, H. R. (1988). The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. Cell 34: 436-444.

Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. Gene. & Dev. 12: 1751-1762.

Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17: 27-41.

Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994). Mechanism of inhibition of Raf-1 by protein kinase A. Molec. Cell. Biol. 14: 6696-6703.

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

Heemskerk, J., and Dinardo, S. (1994). *Drosophila* HEDGEHOG acts as a morphogen in cellular patterning. Cell 76: 449-460.

Herman, M. A., and Horvitz, H. R. (1994). The *Caenorhabditis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. Development 120: 1035-1047.

Huang, E. J., Nocka, K. H., Buck, J., and Besmer, P. (1992).Differential expression and processing of 2 cell associated forms of the Kit-ligand: KL-1 and KL-2. Molec. Biol. Cell. 3: 349-362.

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

Kenyon, C. (1986). A gene involved in the development of the posterior body region of *Caenorhabditis elegans*. Cell 46: 477-487.

Kornfeld, K. (1997). Vulval development in *Caenorhabditis elegans.* TIG 13: 55-61.

Liu, K. S., and Sternberg, P. W. (1995). Sensory regulation of male mating-behavior in *Caenorhabditis elegans*. Neuron 14: 79-89.

Maloof, J. N., and Kenyon, C. (1998). The hox gene *lin-39* is reuired during *C. elegans* vulval induction to select the outcome of Ras signaling. Development 125: 181-190.

McConnell, S. K., and Kaznowski, C. E. (1991). Cell-cycle dependence of laminar determination in developing neocortex. Science 254: 282-285.

Miyazawa, K., William, D. A., Gotoh, A., Nishimaki, J., Broxmeyer, H. E., and Toyama, K. (1995). Membrane-bound steel factor induces more persistent tyrosine kinase activation and longer life span of *c-kit* gene-encoded protein than its soluble form. Blood 85: 641-649.

O'Keefe, L., Dougan, S. T., Gabay, L., Raz, E., Shilo, B. Z., and Dinardo, S. (1997). *spitz* and *wingless*, emenating from distinct borders, cooperate to establish cell fate across the Engrailed domain in the *Drosophila* epidermis. Development 124: 4837-4845.

Reh, T. A., and Cagan, R. L. (1994). Intrinsic and extrinsic signals in the developing vertebrate and fly eye: viewing vertebrate and invertebrate eyes in the same light. Perspect. Dev. Neurobiol. 2:183-190.

Reinke, R., and Zipursky, S. L. (1988). Cell-cell interaction in the *Drosophila* retina: the *bride-of-sevenless* gene is required in photoreceptor R8 for R7 cell development. Cell 55: 321-330.

Rhyu, M. S., Jan, L. Y., and Jan, Y. N. (1994). Asymmetric distribution of NUMB protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76: 477-491.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from *wingless* and *decapentaplegic*. Cell 88: 777-787.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. Cell 50: 649-657.

Rutledge, B. J., Zhang, K., Bier, E., Jan, Y. N., and Perrimon, N. (1992). The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. Genes. & Dev. 6: 1503-1517.

Sawa, H., Lobel, L., and Horvitz, H. R. (1996). The *Caenorhabditis elegans* gene *lin-17*, which is required for certain asymmetric cell divisions, encodes a putative protein similar to seven-transmembrane protein similar to the *Drosophila* Frizzle protein. Gene & Dev. 10: 2189-2197.

Schweitzer, R., Shaharabany, M., Seger, R., and Shilo B. Z. (1995a). Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. Genes. & Dev. 9: 1518-1529.

Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z., and Freeman, M. (1995b). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. Nature, 376: 699-702.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth-factor restricts mammalian neural crest stem-cells to a glial fate. Cell 77: 349-360.

Shah, N. M., Groves, A. K., and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGF-beta superfamily members. Cell 85: 331-343.

Sternberg, P. W., and Horvitz, H. R. (1988). *lin-17* mutations of *Caenorhabditis elegans* disrupt certain asymmetric cell divisions. Dev. Biol. 130: 67-73.

Sternberg, P. W., and Alberola-Ila, J. (1998). Conspiracy theory: RAS and RAF do not act alone. Cell 95: 1-4.

Struhl, G., and Basler, K. (1993). Organizing activity of WINGLESS protein in *Drosophila*. Cell 72: 527-540.

Sulston, J. E., and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. Devel. Biol. 56: 110-156.

Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. Devel. Biol. 78: 577-597.

Sundaram, M., and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. Bioessays 18: 473-480.

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

Wehrle-Haller, B., and Weston, J. A. (1995). Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. Development 121: 731-742.

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. Gene. & Dev. 12: 2445-2462.

Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Stargill, T. W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3', 5'-monophosphate. Science 262: 1065-1069.

Figure 1. Development of the ventral cord precursor cells and tail blast cells in *C. elegans* hermaphrodites and males.

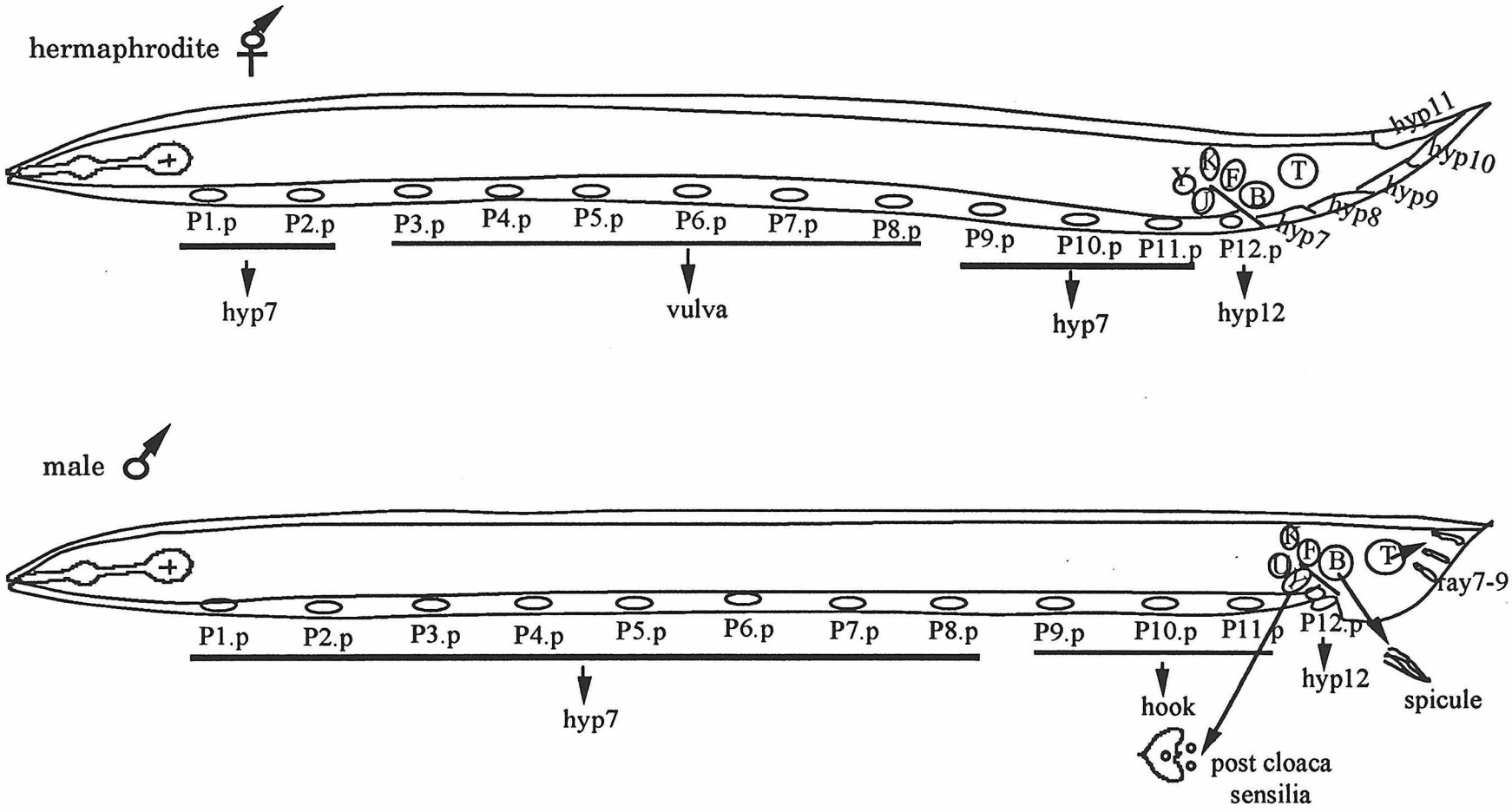

hermaphrodite
hyp11
hyp10
hyp9
hyp8
hyp7
K
F
T
B
U
P1.p
P2.p
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
P9.p
P10.p
P11.p
P12.p
hyp7
vulva
hyp7
hyp12
male
K
F
U
B
T
ray7-9
P1.p
P2.p
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
P9.p
P10.p
P11.p
P12.p
hyp7
hook
hyp12
spicule
post cloaca
sensilia

Figure 2. Multiple signaling pathways mediate several developmental processes in *C. elegans*.

(A). Vulva induction and patterning is mediated by the *lin-3/let-23* EGF signaling pathway, *lin-12/Notch* signaling pathway, and hox genes.

(B). P11/P12 fate specification is controlled by the Wnt signaling pathway, the *lin-3/let-23* EGF signaling pathway, and hox genes.

(C). The hook development in males requires hox genes and *lin-12/Notch* signaling pathway.

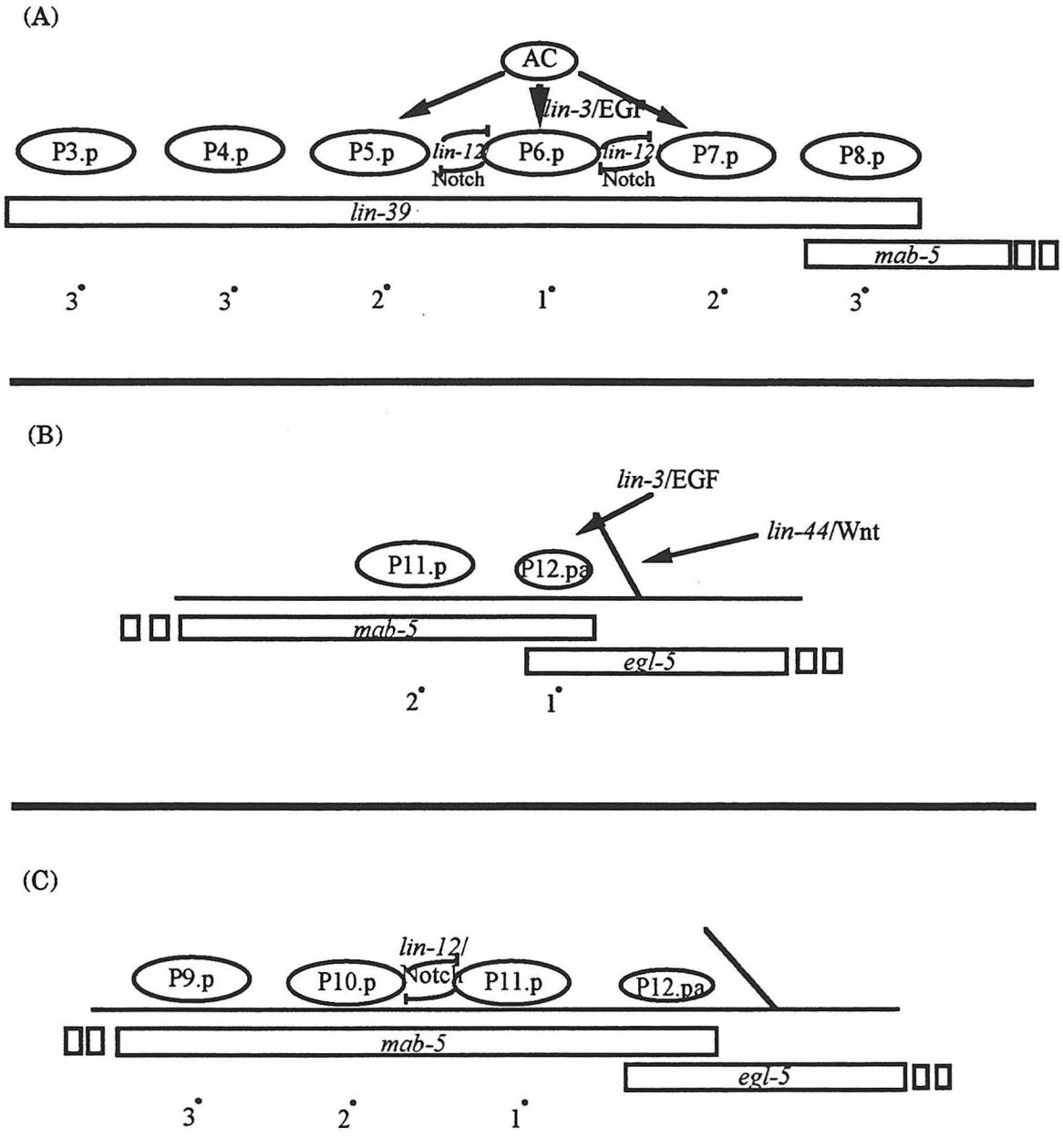
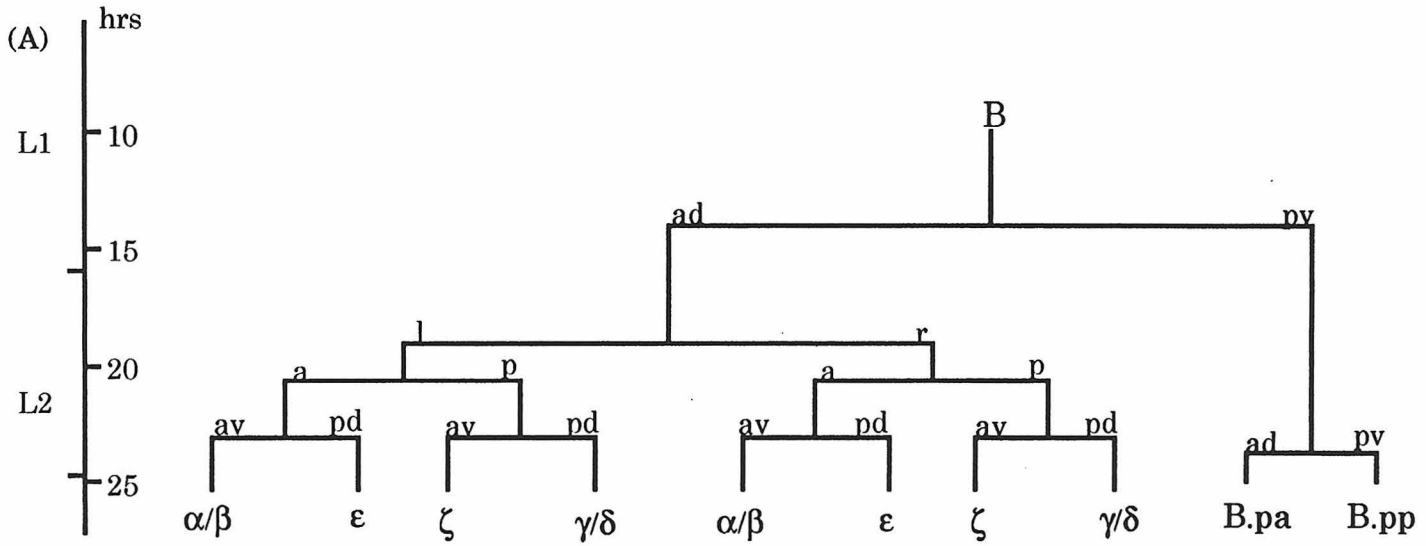
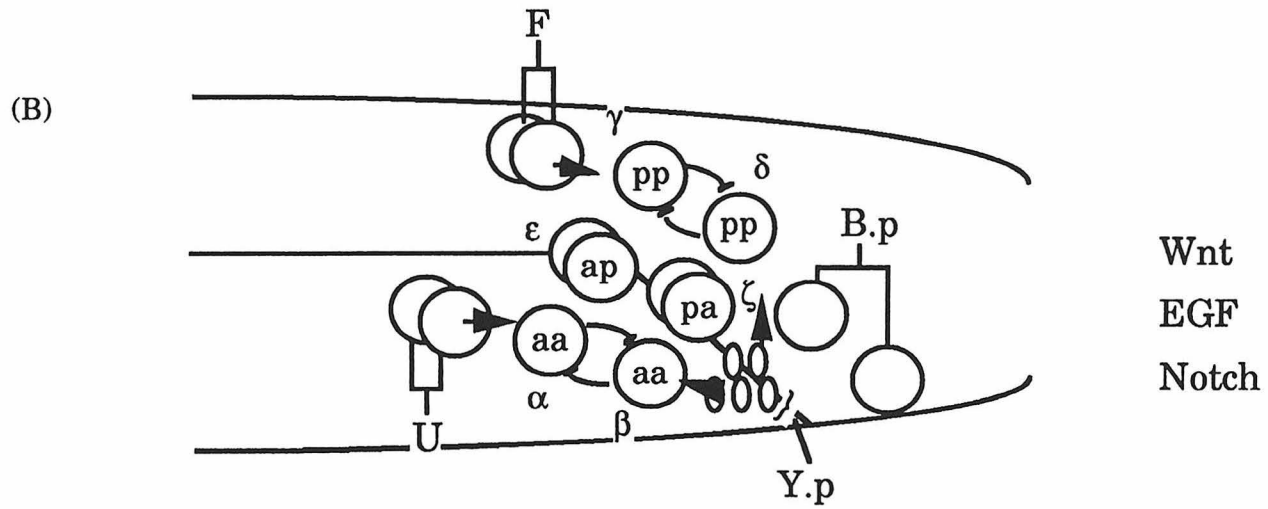

(A)
AC
lin-3/EGF
P3.p
P4.p
P5.p
lin-12
Notch
P6.p
lin-12
Notch
P7.p
P8.p
lin-39
mab-5
3°
3°
2°
1°
2°
3°
(B)
lin-3/EGF
lin-44/Wnt
P11.p
P12.pa
mab-5
egl-5
2°
1°
(C)
lin-12/
Notch
P9.p
P10.p
P11.p
P12.pa
mab-5
egl-5
3°
2°
1°

Figure 3. Early stage B lineage development.

(A). Cell division pattern of the B lineage from 1-cell to 10-cell stage.

(B). Cell positions at B lineage 10-cell stage. Signals from F and U cells promote anterior fates, signals from Y.p cell and its progeny promote posterior fates. Inhibitory interactions between α and β, γ and δ exist. Three signaling pathways, Wnt, *lin-3*/EGF, and *lin-12*/Notch, mediate cell fate specification in early B lineage development.


(A)
hrs
L1
L2
10
15
20
25
B
ad
pv
l
r
a
p
av
pd
α/β
ε
ζ
γ/δ
B.pa
B.pp


(B)
F
γ
pp
δ
ε
ap
B.p
pa
ζ
aa
α
U
β
Y.p
Wnt
EGF
Notch

Chapter II

Interactions of EGF, Wnt, and HOM-C genes specify the P12 neuroectoblast fate in *C. elegans*

Lily I. Jiang and Paul W. Sternberg

Published in *Development* **125**, 2337-2347 (1998).

(reprinted with permission)

Development 125, 2337-2347 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5178

Interactions of EGF, Wnt and HOM-C genes specify the P12 neuroectoblast fate in *C. elegans*

Lily I. Jiang and Paul W. Sternberg*

Howard Hughes Medical Institute and Division of Biology, California Institute of Technology, Mail Code 156-29, Pasadena, CA 91125, USA

*Author for correspondence (e-mail: PWS@cco.caltech.edu)

Accepted 3 April; published on WWW 19 May 1998

SUMMARY

We investigate how temporal and spatial interactions between multiple intercellular and intracellular factors specify the fate of a single cell in *Caenorhabditis elegans*. P12, which is a ventral cord neuroectoblast, divides postembryonically to generate neurons and a unique epidermal cell. Three classes of proteins are involved in the specification of P12 fate: the LIN-3/LET-23 epidermal growth factor signaling pathway, a Wnt protein LIN-44 and its candidate receptor LIN-17, and a homeotic gene product EGL-5. We show that LIN-3 is an inductive signal sufficient to promote the P12 fate, and the conserved EGF signaling pathway is utilized for P12 fate specification; *egl-5* is a downstream target of the *lin-3/let-23* pathway in specifying P12 fate; and LIN-44 and LIN-17 act synergistically with *lin-3* in the specification of the P12 fate. The Wnt pathway may function early in development to regulate the competence of the cells to respond to the LIN-3 inductive signal.

Key words: EGF, HOM-C, Wnt, Cell fate specification, Gene interactions, *Ceanorhabditis elegans*

INTRODUCTION

In certain developmental fields, cells are exposed to multiple extrinsic signaling molecules that combine with intrinsic factors to control cell proliferation, differentiation, and morphogenesis. Although we have an increasing understanding of how a single factor regulates cellular behaviors, we have limited understanding of how multiple factors work in concert to ensure cells take on their correct fates. Here we investigate how an epidermal growth factor (EGF)-like protein, a Wnt protein, and a homeotic cluster (HOM-C) transcription factor interact to specify P12 neuroectoblast fate in *Caenorhabditis elegans*.

The cells P11 and P12 are the most posterior pair of postembryonic ventral cord precursors (Sulston and Horvitz, 1977). These two cells both have the potential to express P12 fate during the early first larval (L1) stage before they enter the ventral cord (Sulston and White, 1980). When either cell is ablated by laser microsurgery, the remaining one takes on P12 fate. How is the P12 fate specified? Though no systematic studies on P11/P12 cell fate specification have been reported, previous investigations have accumulated data indicating that several classes of genes are involved in P11/P12 cell fate specification. These include genes of the *lin-3/let-23* pathway, which is the *C. elegans* EGF signaling pathway, a Wnt family gene *lin-44*, and a HOM-C gene *egl-5*.

These three classes of genes have been primarily studied for reasons other than P11/P12 cell fate specification. The *lin-3/let-23* signaling pathway, which is similar to the EGF signaling pathway of mammals (Fantl et al., 1993), has been studied extensively in *C. elegans* vulval development (reviewed by Sundaram and Han, 1996; Kornfeld, 1997). *lin-3* encodes a membrane-spanning protein with a single extracellular EGF domain that is similar in structure to members of the EGF family of growth factors (Hill and Sternberg, 1992). *let-23* encodes a receptor tyrosine kinase of the EGF receptor family (Aroian et al., 1990). LIN-3 functions as an intercellular signal and its activity is both necessary and sufficient for vulval induction (Ferguson and Horvitz, 1985; Hill and Sternberg, 1992; Katz et al., 1995), while LET-23 is the likely receptor for the LIN-3 signal. LET-23 transduces LIN-3 activity via an SH2/SH3 adaptor protein SEM-5 (Clark et al., 1992a), which in turn activates the downstream RAS protein LET-60 (Han and Sternberg, 1990). *lin-15* defines a general inhibitory pathway that negatively regulates *let-23* activity and prevents cells undergoing vulval differentiation (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Herman and Hedgecock, 1990; Clark et al., 1994; Huang et al., 1994). The *lin-3/let-23* signaling pathway is also utilized to specify certain cell fates in male spicule development (Chamberlin and Sternberg, 1994).

The *C. elegans* gene *lin-44* is a member of the Wnt family genes (Herman et al., 1995). Wnt family genes encode secreted glycoproteins and are implicated in intercellular signaling to control cell fate specification and cell proliferation in many organisms (reviewed by Nüsse and Varmus, 1992; Moon et al., 1997). In *C. elegans*, while the major defects in *lin-44* mutants appear to be the reversed polarities of certain asymmetric cell divisions in the tail region, they are also defective in the specification of P12 fate (Herman and Horvitz, 1994). Both in

situ hybridization and the analysis of reporter gene constructs indicated that *lin-44* is expressed in epidermal cells at the tip of the tail and posterior to the cells affected by *lin-44* mutation (Herman et al., 1995). Mosaic analysis demonstrated that *lin-44* acts cell nonautonomously and *lin-44* function is required in the cells in which *lin-44* expression is observed. *lin-17* encodes a protein with seven putative transmembrane domains, homologous to the *Drosophila* Frizzled protein (Sawa et al., 1996). *lin-17* regulates several asymmetric cell divisions and cell fates which are also controlled by *lin-44* (Sternberg and Horvitz, 1988), therefore has been proposed to be a receptor for the Wnt protein LIN-44 (Sawa et al., 1996).

HOM-C genes are conserved throughout metazoan evolution. These HOM-C transcription factors establish position-specific fates along body axes (Lewis, 1978; reviewed by Krumlauf, 1994). HOM-C genes can act in multiple cell types and at multiple time during development (reviewed by Lawrence and Morata, 1994; Salser and Kenyon, 1994). They are regulated by multiple intercellular and intracellular molecules (Immergluck et al., 1990). They also regulate diverse targets to control position-specific cell fates (Mastick et al., 1995). In *C. elegans* a cluster of four HOM-C genes has been identified. Along the anteroposterior body axis it consists of *lin-39*, *mab-5*, and *egl-5*, respective homologues of the *Drosophila* genes *Sex combs reduced/deformed/proboscipedia*, *Antennapedia*, and *Abdominal-B*, with a labial homologue, *ceh-13*, being between *lin-39* and *mab-5* (reviewed by Salser et al., 1994). *lin-39* activity is required in the central body region for vulval differentiation and patterning, while *mab-5* and *egl-5* are involved in cell fate specification of the tail region (Kenyon 1986; Chisholm, 1991; Clark et al., 1993; Salser et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998).

We are particularly interested in these three classes of genes because mutations in all these genes show P11/P12 cell fate specification defects. We sought to determine how these intrinsic and extrinsic factors act together to specify a certain cell fate. We first demonstrate that LIN-3 is an inductive signal for the P12 fate, and the *lin-3/let-23* pathway functions to specify P12 fate in a similar way to its function during vulval induction. Then we examine whether the HOM-C gene, *egl-5*, sets up the competence of the cells to the LIN-3 inductive signal by establishing a pre-pattern, or if it plays an instructive role in P11/P12 cell fate specification. Our results support the latter possibility, that *egl-5* is a downstream target of the *lin-3/let-23* pathway and its activity is required in executing P12 fate. Finally we explore the interactions between the *lin-3/let-23* and the Wnt signaling pathways. Our results suggest that *lin-44* and *lin-3* may act at different times during development corresponding to two genetically parallel pathways, and the Wnt pathway may regulate the competence of the P11/P12 cells to respond to the LIN-3 signal.

MATERIALS AND METHODS

General methods

Routine culturing, maintenance and genetic manipulations of *C. elegans* strains were performed according to standard procedures (Brenner, 1974). N2 (Bristol) strain was used as wild type (Brenner, 1974). The following strains were used.

LG I: *lin-17(n671)* (Ferguson and Horvitz, 1985), *lin-44(n1792)* (Herman and Horvitz, 1994).

LG II: *let-23(sy97)* (Aroian and Sternberg, 1991).

LG III: *egl-5(n945)* (Chisholm, 1991).

LG IV: *dpy-20(e1282)* (Brenner, 1974), *lin-3(n378)* (Ferguson and Horvitz, 1985), *lin-3(n1059)* (Ferguson and Horvitz, 1985), *let-60(sy93)* (Han et al., 1990).

LG X: *lin-15(e1763)* (Ferguson and Horvitz, 1985), *sem-5(n2019)* (Clark et al., 1992a).

Transgenic strains:

PS1427: *unc-31(e169); syIs6[unc-31(+), pRH51(hsLIN-3EGF) 50 ng/μl]* (Katz et al., 1995).

PS2467: *dpy-20(e1282); syEx178[dpy-20(+), pLJ5(hsEGL-5) 20 ng/μl]*.

PS2347: *dpy-20(e1282); muIs13[rol-6(su1006), egl-5-lacZ] him-5(e1490)* (Wang et al., 1993).

Construction of *hsp16.1-egl-5* transgene

The *hsp16.1-egl-5* construct, pLJ5, was constructed in the same way as previously described constructs *hs-mab-5* and *hs-lin-39* (Salser and Kenyon, 1992; Hunter and Kenyon, 1995). Briefly, primers overlapping sequences of the start and stop codons were used to amplify the coding sequence of *egl-5* from an *egl-5* cDNA clone by PCR. The amplification products were sequenced and ligated into the *HpaI* site of the *hsp16.1* gene (Russnak and Candido, 1985). This construct generates a fusion protein with the first 44 amino acids of *hsp16.1* gene product and the full length EGL-5 protein.

Construction of transgenic strains

Transgenic animals were generated by microinjection of DNA into the mitotic germline of hermaphrodites according to the method of Mello et al. (1991). *syEx178* was obtained by microinjection of pLJ5 plasmid at 20 ng/μl, pMH86 (dpy-20(+)) at 15 ng/μl, and carrier DNA (pBlueScript) at 120 ng/μl into *dpy-20(e1282)* mutant animals. To test the function of the construct, the transgenic line *syEx178* was crossed into *egl-5(n945); dpy-20(e1282)* and *let-23(sy97) unc-4(e120)/mnC1(dpy-10 unc-52); dpy-20(e1282)* strains. The non-Dpy phenotype indicates the presence of the transgene.

syIs6 was obtained by X-ray integration of the *syEx23* strain, which expresses the EGF domain of LIN-3 under the heat shock promoter and has been described by Katz et al. (1995).

muIs13 is an integrated strain containing the *egl-5-lacZ* transgene (Wang et al., 1993), kindly provided by Craig Hunter. For ease of scoring, *dpy-20(e1282)* mutation was crossed into the strain to suppress the Roller phenotype caused by the transformation marker.

syIs6; dpy-20(e1282); muIs13 him-5(e1490) strain was constructed by crossing *syIs6; dpy-20(e1282); him-5(e1490)* males with *muIs13 him-5(e1490)* hermaphrodites. Animals homozygous for *syIs6* and *muIs13* displayed 100% multivulva phenotype under heat shock condition and had 100% β-galactosidase-positive staining.

The assay for β-galactosidase activity was performed as described previously (Fire et al., 1990).

Heat shock of transgenic animals

Cohorts of adult hermaphrodites were placed on standard worm culture plates to collect eggs. The hermaphrodites were transferred to a fresh plates every hour. The age of the worms at heat shock was determined as hours after egg-lay. Different strains hatch at slightly different times (±2 hours after egg-lay), and times were normalized to hatching. Animals were heat shocked in a 33°C water bath for 30 minutes or 60 minutes as indicated in each figure legend. For the experiment involving EGL-5 overexpression in a *let-23(sy97)* mutant background, eggs were collected by cutting egg-laying defective adult hermaphrodites into halves and then heat shocking at certain developmental times.

Anatomical and cell lineage analysis

L3 or early-mid L4 hermaphrodites were examined with Nomarski optics. P11 and P12 fates were determined according to the distinct nuclear morphologies and positions of P11.p and P12.pa cells. Cell

lineages were followed at 20°C as described by Sulston and Horvitz (1977).

RESULTS

P11/P12 development

In *C. elegans* there are twelve ventral cord precursor cells, P1-P12, numbered from anterior to posterior along the body axis. These cells divide postembryonically to generate cells of the ventral nervous system, as well as the vulva (Sulston and Horvitz, 1977). P11/P12 are the most posterior pair of the ventral cord precursors. At hatching, the cells AB.plapappa (left side) and AB.prapappa (right side) are disposed laterally (Sulston et al., 1983). In hermaphrodites, they start to migrate ventrally several hours after hatching and enter the ventral cord about 8-9 hours after hatching at 20°C (Fig. 1A). The left cell migrates anteriorly and becomes P11, whereas the right cell migrates posteriorly and becomes P12. Two hours later they both divide once. The anterior daughters, P11.a and P12.a are neuroblasts that will divide for three more rounds to generate several ventral cord neurons. These neurons are morphologically indistinguishable under Nomarski optics. The posterior daughter of P11, P11.p, does not divide but rather fuses with the large epidermal syncytium hyp7. P12.p divides once more about 1 hour prior to L1 molt to generate P12.pa, which becomes a unique epidermal cell, hyp12, and P12.pp, which undergoes cell death. P11.p and P12.pa can be distinguished by their different nuclear morphologies and positions observed with Nomarski optics (Fig. 1B). Sulston and White (1980) demonstrated that prior to

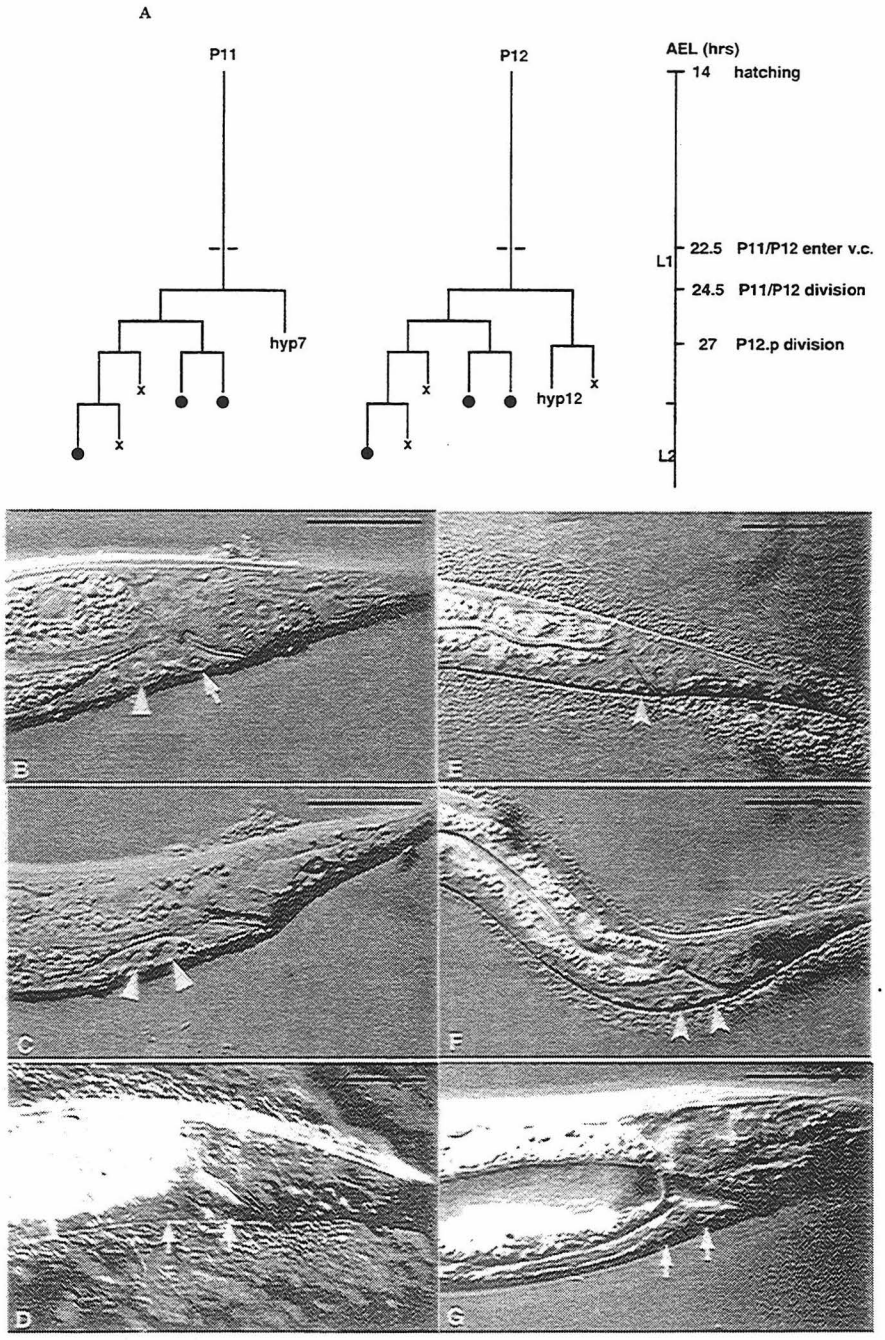


Fig. 1. P11/P12 phenotype observed in Nomarski photomicrographs. (A) Wild-type P11/P12 lineages at 20°C (Sulston and Horvitz, 1977). Terminal fates are: •, ventral cord neuron; x, programmed cell death. Timing is indicated as AEL, denoting hours after egg-lay. v.c. denotes ventral cord. (B) Wild type. (C) *let60(sy93dn)*. (D) *syIs6*. (E) wild-type animal, P12.pp undergoes cell death, indicated by a concave arrowhead. (F) P11 and P12 lineages are followed after heat shock treatment of *syIs6* strain (n=4). P11.p divides as does P12.p, and both P11.pp and P12.pp die. (G) *syEx178*. In B-D and G, arrowhead indicates P11.p, arrow points to P12.pa. Scale bars, 20 μm.

migration both P11 and P12 cells are able to express the P12 fate; and if only a single cell is present, it will adopt the P12-like fate. Therefore P12 represents a primary fate, while P11 is a secondary fate.

Mutants of several *lin-3/let-23* pathway components have defects in P11/P12 cell fate specification

Mutants of several *lin-3/let-23* pathway components have been reported to display defects in P11/P12 cell fate specification. Loss-of-function alleles of *let-23* showed a loss of the cell P12.pa with concommitant duplication of P11.p in the hermaphrodite tail (Fixsen et al., 1985; Aroian and Sternberg, 1991). Lineage analysis in males indicated that this defect likely represents a transformation of P12 to P11 fate, as the anterior branch is also affected (Fixsen et al., 1985). Mutations at the *lin-15* locus, which encodes negative regulators of the *lin-3/let-23* pathway, have the opposite defect, P11 to P12 cell fate transformation (Fixsen et al., 1985). Other components, *sem-5* and *let-60*, which encode a SH2/SH3 domain protein (Clark et al., 1992a) and a RAS protein (Han and Sternberg, 1990), respectively, are also involved in P11/P12 cell fate specification (Clark et al., 1992b). We examined hermaphrodites homozygous for a reduction-of-function allele of *sem-5*, *n2019*, and found a 31% penetrant P12 to P11 cell fate transformation (Table 1A). A dominant-negative allele of *let-60*, *sy93*, also displays a 45% penetrant P12 to P11 fate transformation (Fig. 1C, Table 1A).

Although mutations in these *lin-3/let-23* pathway components result in a P11/P12 defect, the penetrance of this phenotype is incomplete. We propose two possible models. The *lin-3/let-23* pathway functions in additional capacities including vulval induction, male spicule development, hermaphrodite fertility, and L1 lethality (Aroian and Sternberg, 1991). Although functions such as vulval induction, male spicule development, and hermaphrodite fertility can be genetically separated (Aroian et al., 1994; Lesa and Sternberg, 1997), mutations with P11/P12 defect share a common L1 lethality phenotype. It is possible that the P11/P12 defect and the lethality phenotype require similar aspects of gene function (e.g., expression during the L1 stage), therefore the dead larvae with P11/P12 defect are excluded from scoring. Alternatively, there might exist another pathway acting in conjunction with the *lin-3/let-23* pathway for P11/P12 fate specification.

Given the fact that these *lin-3/let-23* pathway components are involved in P11/P12 cell fate specification, we asked if the genetic interactions among these genes are conserved. During vulval development, *lin-15* defines a general inhibitory pathway which negatively regulates *let-23* activity. As *let-23* and *lin-15* also have an opposite effect on P11/P12, we tested if *lin-15* acts at the level of *let-23*. We constructed a strain defective in both genes. 30% of *let-23; lin-15* double mutants display a P12 to P11 cell fate transformation (Table 1A), resembling the mutation in *let-23* alone. Thus *let-23* is epistatic to *lin-15*, reminiscent of *lin-15* negative regulation of *let-23* in vulval induction.

LIN-3 is an inductive signal sufficient for P12 fate

Since none of the existing *lin-3* mutants exhibits any defect in P11/P12 cell fate specification and null alleles of *lin-3* are L1 lethal, it has been difficult to determine if *lin-3* plays a role in P11/P12 cell fate specification. We circumvented this problem by testing whether overexpression of LIN-3 would affect P11

Table 1.

A. *let-23* pathway is involved in P11/P12 cell fate specification.

Genotype*	*n*†	% P11→P12‡	% P12→P11§
N2 (wild type)	50	0	0
let-23(lf)	50	0	44
lin-15(lf)	74	54	0
let-23(lf); lin-15(lf)	49	0	33
sem-5(lf)	45	0	31
let-60(dn)	51	0	45

B. *egl-5* is epistatic to the lin-*3/let-23* pathway in P12 fate specification.

Genotype*	*n*†	% P11→P12‡	% wild type	% P12→P11§
egl-5(lf)	63	0	0	100
egl-5(lf); lin-15(lf)	41	0	0	100
egl-5(lf); let-23(lf)	25	0	0	100
syIs6, non-hs	50	0	100	0
egl-5(lf); syIs6 , non-hs	50	0	0	100
egl-5(lf); syIs6¶, hs	37	0	0	100
let-23(lf)	50	0**	56††	44‡‡
let-23(lf); syEx178, hs	21	15**	75††	10‡‡

non-hs, animals were not treated with heat shock; hs, animals were heat shocked.

*Alleles used are: *let-23(sy97)*, a severe reduction-of-function allele; *lin-15(e1763)*, a severe reduction-of-function allele, *sem-5(n2019)*, a reduction-of-function allele; *let-60(sy93)*, a dominant-negative allele; *egl-5(n945)*, a severe reduction-of-function. *syIs6* is a transgenic strain bearing the *hsLIN-3EGF* construct, *syEx178* is a transgenic strain bearing the *hsEGL-5* construct.

†Number of animals examined.

‡Percentage of animals with two P12-like cells. Animals were scored under Nomarski at L3-L4 stage, cell fate transformation is determined by nuclear morphology and position.

§Percentage of animals with two P11-like cells.

¶Animals were heat shocked during embryogenesis and L1 stage in 33°C water bath for 30 minutes.

**P=0.0223, Fisher's Exact Test

††P=0.7935, Fisher's Exact Test

‡‡P=0.0057, Fisher's Exact Test

and P12. A transgenic strain bearing a construct that expresses the EGF domain of LIN-3 under the control of a heat shock promoter/enhancer was used for this experiment. This transgene, the 63 amino acid EGF domain of LIN-3 driven by the inducible and tissue general *hsp16-41* heat shock promoter (Stringham et al., 1992), has been demonstrated to be functional and sufficient to induce vulval development (Katz et al., 1995). The transgenic strain *syIs6* carries a high dose of the *hslin-3EGF* plasmid integrated in the genome. When these transgenic animals are grown at 25°C, the basal activity of LIN-3EGF can induce a multivulva phenotype, whereas the P11 and P12 cells remain wild type. We carried out a time course study to induce the expression of LIN-3EGF during embryogenesis and the L1 stage by a pulse of heat shock treatment at certain developmental times (Fig. 2A). We first observed that overexpression of LIN-3EGF does lead to a duplication of the cell P12.pa with a concommitant loss of P11.p in the hermaphrodite tail (Fig. 1D). We interpret this as a gain-of-function *lin-3* phenotype by analogy with results from the vulva and the spicules (Katz et al., 1995; Chamberlin and Sternberg, 1994). We confirmed this phenotype as being the result of the P11 to P12 cell fate transformation by following the P11/P12 lineages in hermaphrodites after heat shock (n=4). As shown in Fig. 1F, P11.p behaves like P12.p: it divides an extra round, and P11.pp undergoes cell death as does P12.pp. Also, the P11 to P12 cell fate transformation occurs when overexpression of LIN-3EGF is induced at late embryogenesis; it peaks to almost 100% when induced within several hours after hatching. Later, the effect of LIN-3EGF tapers off; overexpression of LIN-3EGF does not have any effect on P11/P12 cell fate after the P11/P12 cells enter the ventral cord. This timing is consistent with the ablation results of Sulston and White (1980) who found that the P11 cell loses its competence to become P12 after it enters the ventral cord. We conclude that LIN-3 is sufficient to induce the P12 fate and infer that LIN-3 is an inductive signal for the P12 fate.

egl-5 acts in a common pathway with the *lin-3/let-23* pathway in P12 fate specification

The *C. elegans* HOM-C gene *egl-5* is a homolog of the *Drosophila Abdominal-B* gene and is important for patterning the tail region of *C. elegans* (Chisholm, 1991; Wang et al., 1993). Loss-of-function mutations in *egl-5* cause a P12 to P11 cell fate transformation, indicating that *egl-5* activity is required for the P12 fate (Kenyon,

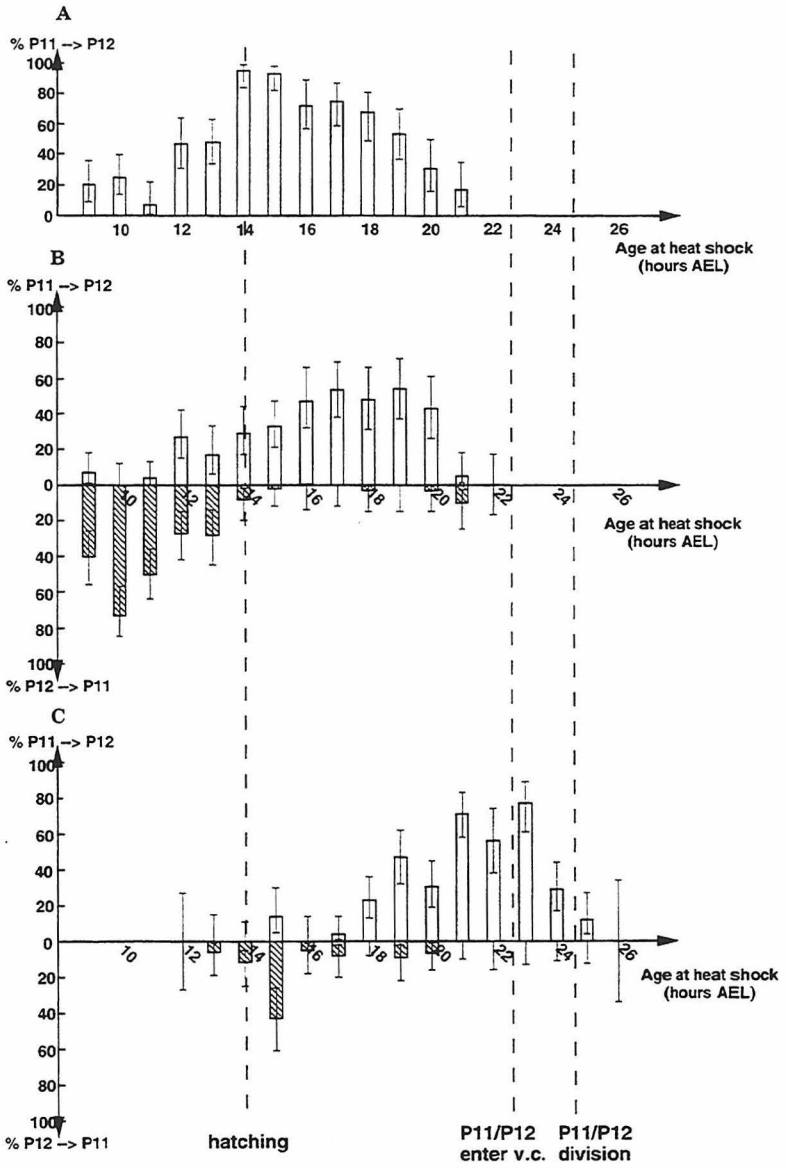


Fig. 2. Time course of the effect of overexpression of LIN-3EGF and EGL-5 on P11/P12 fate specification. (A) *syIs6(hsLIN-3EGF)*. (B) *syIs6(hsLIN-3EGF)*; *lin-44(n1792)*. (C) *syEx178(hsEGL-5)*. Eggs were collected every hour on fresh plates and heat shocked at certain times after egg-lay as indicated on the abscissa. AEL denotes after-egg-lay. The heat shock conditions were 33°C, 30 minutes for A and B, and 33°C, 60 minutes for C. Open bars indicate percentage of P11 to P12 transformation; hatched bars indicate P12 to P11 transformation. Error bars are 95% confidence limits based upon the binomial distribution. Dashed lines indicate the times of hatching, P11/P12 cells entering the ventral cord, and the first division of P11/P12 cells, which are about 14, 22.5, and 27 hours after egg-lay respectively.

1986; Chisholm, 1991). We examined the epistasis relationship between *egl-5* and mutations of the *lin-3/let-23* pathway. Doubly mutant strains were constructed bearing an *egl-5* mutation and several *lin-3/let-23* pathway components. *egl-5(n945)* mutants display a 100% penetrance of P12 to P11 transformation. All the double mutants behave like mutation in *egl-5* alone (Table 1B). For example, *lin-15(lf)* animals display a 54% penetrance of P11 to P12 fate transformation, while all *egl-5(lf); lin-15(lf)* double mutants show a P12 to P11 fate transformation. Moreover, when overexpression of LIN-3EGF is induced in *egl-5* mutant animals, its effect on P11/P12 is completely blocked by *egl-5* mutation (Table 1B). Thus *egl-5* acts in a common pathway with the *lin-3/let-23* pathway in P12 fate specification.

egl-5 plays an instructive rather than permissive role in P12 fate specification

Previous studies have shown that HOM-C genes can specify the competence of cells to respond to an intercellular signal, thus establishing a pre-pattern in a developmental field (Clandinin et al., 1997). They themselves can also be regulated by intercellular signaling molecules (e.g., Immergluck et al., 1990; Maloof and Kenyon, 1998). *egl-5* might play a permissive role in P12 fate specification by setting up the competence of the cell to respond to the LIN-3 inductive signal, or *egl-5* might be an instructive factor for P12 fate specification. To distinguish these hypotheses, we first tested if overexpression of EGL-5 has an effect on P11/P12 cell fate specification. We engineered the full length *egl-5* cDNA (Salser and Kenyon, 1992) under the control of the *hsp16.1* promoter. Transgenic animals bearing this pLJ5 construct as an extrachromosomal array, *syEx178*, were generated. Upon heat shock treatment, the *hsp16.1* promoter should drive EGL-5 expression ubiquitously in somatic tissues (Russnak and Candido, 1985). When *syEx178* was introduced into *egl-5(n945)* mutants, expression of the transgene was able to rescue the P11/P12 mutant phenotype (data not shown). Overexpression of EGL-5 has a dominant sterile phenotype (L. Jiang, unpublished observation), making it impossible to examine the rescue of the egg-laying behavior. To analyze the effect of EGL-5 in wild-type animals, a pulse of EGL-5 expression was induced in the transgenic strain during late embryogenesis and L1 stage (Fig. 2C). Overexpression of EGL-5 in wild-type animals does lead to a P11 to P12 fate transformation (Fig. 1G). The most effective time to cause a P11 to P12 fate transformation is when overexpression of EGL-5 is induced around the time of the entry of P11/P12 into the ventral cord. This is later than the time of maximal effect of LIN-3EGF overexpression, which is within several hours after hatching. This temporal difference in responsiveness is consistent with our observation that *egl-5* is downstream of the *lin-3/let-23* pathway. It is also consistent with the observation that *egl-5* is not expressed in P cells during the early L1 stage (Wang et al., 1993).

We then asked if overexpression of EGL-5 could suppress the P11/P12 defect of *let-23* mutants. If *egl-5* is a permissive factor for P12 fate, no rescue of the P11/P12 defect would be expected; whereas if *egl-5* is an instructive factor, overexpression of EGL-5 should be able to rescue the P11/P12 defect caused by *let-23* mutation, and may additionally result in a P11 to P12 cell fate transformation. 44% of *let-23(sy97)* animals show a P12 to P11 transformation and 56% are wild type. When overexpression of EGL-5 is induced in transgenic *let-23(sy97)* animals bearing *syEx178*, the percentage of animals with a P12 to P11 transformation is lowered to 10% (P=0.0057, Fisher's Exact Test), while the percentage of wild-type animals is increased to 75%, the remaining 15% animals display a P11 to P12 cell fate transformation (P=0.0223) (Table 1B). This result suggests that overexpression of EGL-5 suppresses the P11/P12 defect of *let-23* mutants, as predicted by the instructive model. Therefore *egl-5* plays an active role in P12 fate specification and acts downstream of *let-23*.

The effect of overexpression of EGL-5 in a *let-23* deficient background is not as prominent as it is in a wild-type background. This may be attributed to technical limitation: *let-23(sy97)* worms are egg-laying defective so that mixed stage animals were collected for the heat shock experiments and timing may have been imprecise. Another possibility is that there exists a feedback loop between *egl-5* and the *lin-3/let-23* pathway.

egl-5 is a downstream target of the *lin-3/let-23* pathway in specifying P12 fate

We next tested whether *egl-5* is a downstream target of the *lin-3/let-23* pathway for P12 fate specification. Expression of an *egl-5-lacZ* reporter gene is detected in the cell P12.pa. As overexpression of LIN-3EGF leads to a P11 to P12 cell fate transformation, we examined if the transformed P11.pa cell is

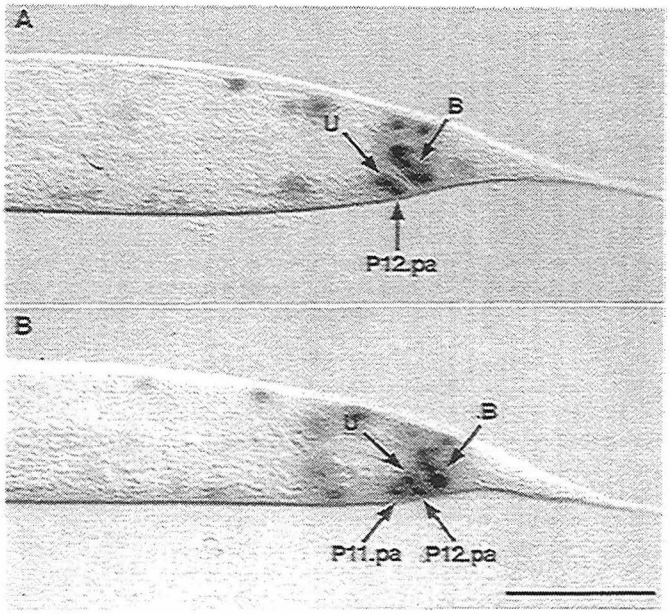


Fig. 3. LIN-3 can induce *egl-5* transcription in P12 lineage. (A) In wild-type animals, *egl-5-lacZ* expression is detected in P12.pa cell. (B) When overexpression of LIN-3EGF induces P11 to P12 transformation, *egl-5-lacZ* expression is detected in both P11.pa and P12.pa cells. Scale bar, 20 μm.

able to express the *egl-5-lacZ* marker. A strain bearing both the *hs-lin-3EGF* and the *egl-5-lacZ* transgenes was constructed. When overexpression of LIN-3EGF is induced at the proper time based on results of Fig. 2A, which is 15-17 hours after eggs were laid, a P11 to P12 cell fate transformation occurs in about 70% animals. As shown in Fig. 3, *egl-5-lacZ* expression is turned on in both the normal P12.pa and the transformed P11.pa cells. This observation indicates that *egl-5* expression in P12.pa can be turned on upon activation of the *lin-3/let-23* pathway.

Does *lin-44* act via *lin-3*?

lin-44, a gene encoding a Wnt family protein (Herman et al., 1995), is a member of a third class of genes that affect P11/P12 cell fate specification (Herman and Horvitz, 1994). 16% of *lin-44(n1792)* mutant animals show a P12 to P11 cell fate transformation. We wondered how the two signaling molecules, LIN-3 and LIN-44, act together to specify P12 fate. One possibility is that *lin-44* regulates *lin-3* expression by controlling the identity of the cell that normally expresses LIN-3 in the tail region. There are several reasons for this hypothesis. First, several cells in the tail region are defective in *lin-44* mutants. These include B, F, U, and T cells, which are posterior to the P11/P12 cells and anterior to *lin-44* expressing cells hyp8-11 (Herman et al., 1995). Second, analysis of *lin-3-lacZ* reporter constructs suggests that a potential source of LIN-3 signal exists in the tail region posterior to the P11/P12 cells (R. Hill, C. Chang and P. Sternberg, unpublished observation). Third, it has been suggested that Wnt genes are involved primarily in short-range cell interactions (van den Heuvel et al., 1989; Vincent and Lawrence, 1994). P11 and P12 lie anterior to the cells affected by *lin-44*, and may not be in direct contact with the *lin-44* expressing cells. Fourth, *lin-44* expression is turned on during embryogenesis, which is prior to *lin-3* expression in the tail.

If *lin-44* only regulates *lin-3* expression, overexpression of LIN-3EGF should be able to rescue the P11/P12 defect in *lin-44* mutants and have the same effect as it does in wild-type background. To test this hypothesis we crossed the *syIs6* strain into *lin-44(n1792)* mutant background and conducted heat shock experiments. As shown in Fig. 2B, overexpression of LIN-3EGF at early L1 stage rescues the P12 to P11 transformation caused by loss of *lin-44* function and results in a P11 to P12 transformation. However, the effect of LIN-3EGF overexpression is not as robust as it is in a wild-type background. In a *lin-44* mutant background, overexpression of LIN-3EGF only results in a maximum 50% P11 to P12 transformation, whereas in a wild-type background the transformation is almost 100% at the same stage. This difference is not expected if *lin-3* expression is solely regulated by *lin-44* in the tail region. However, we cannot rule out the possibility that the cells that provide functional LIN-3 might be altered in *lin-44* mutants, and thus overexpression of LIN-3EGF in *lin-44* mutants might not be as effective as it is in wild type.

To further test the hypothesis that *lin-44* regulates *lin-3*, we did the following two experiments. First, we examined the expression pattern of a *lin-3-lacZ* reporter gene in a *lin-44* mutant background. Transgenic animals bearing a *lin-3-lacZ* reporter construct which contains 12 kb of *lin-3* genomic sequence have been generated (R. Hill, C. Chang, and P. Sternberg, unpublished observations). *lin-3-lacZ* is expressed in the hermaphrodite tail region in newly hatched larvae. We believe the expression resides in the cells K, K′, and the daughters of K, which are posterior to the P11/P12 cells. When the transgenic strain is crossed into the *lin-44(n1792)* mutant background, we did not observe any change in the level of expression nor of the position of the cells (n=45).

Second, we examined the P11/P12 phenotype in *lin-44; lin-15* double mutant animals. *lin-15* is a negative regulator of the *lin-3/let-23* pathway and acts at the level of *let-23* in vulval development (Huang et al., 1994). *lin-15(e1763)* mutant animals display a 54% penetrance of P11 to P12 fate transformation (Table 1A). However *lin-15* acts in a signal-dependent manner as loss of LIN-3 activity removes its effect on P11 and P12 cells. *lin-3(n378/n1059)* mutants do not display a P11/P12 defect (Table 2), neither do *lin-3(n378/n1059); lin-15(e1763)* double mutant animals (n=45). If *lin-44* solely regulates *lin-3* activity, we would expect a similar result. Yet double mutant animals defective in both *lin-44* and *lin-15* show a mixed cell fate transformation, 10% P12 to P11 fate transformation and 35% P11 to P12 fate transformation (n=116). This cannot result from a quantitative effect since the *lin-44* genotype has a more severe effect on P12 than the *lin-3* genotype used. This additive effect argues against the hypothesis that *lin-44* affects P11/P12 fate by regulating *lin-3* activity. An additional *lin-44* function is required for proper P11/P12 fate specification.

lin-3 and *lin-44* act synergistically in P12 fate specification

We next tested the interactions between *lin-3* and *lin-44* by examining the P11/P12 defect in a strain defective in both genes. Heterozygotes of a *lin-3* reduction-of-function allele, *n378*, in *trans* to a null allele, *n1059*, do not display the P11/P12 defect, but have almost no vulval induction and severely defective spicules. However, *n378/n1059* enhances the phenotype of *lin-44(n1792)* from 16% P12 to P11 transformation to 78% (P<0.0001) (Table 2). Although it does not rule out a sequential model where *lin-3* acts downstream of *lin-44*, the strong synergy between *lin-3* and *lin-44* is consistent with the two signals acting in parallel. To confirm the interaction between *lin-3* and *lin-44*, we tested if synergy exists between *lin-3* and *lin-17* mutations. *lin-17*, which encodes a putative seven-transmembrane protein similar to the *Drosophila* Frizzled protein, has been suggested to be a receptor for the LIN-44 protein (Sawa et al., 1996). *lin-17(n671)* animals have a penetrance of 29% P12 to P11 fate transformation (Table 2). Double mutant animals defective in both *lin-17* and *lin-44* display a similar defect to *lin-17(n671)* alone, showing 28% P12 to P11 fate transformation (P=1) (Table 2). This lack of synergy supports the hypothesis that LIN-44 signal acts through the receptor LIN-17 for P11/P12 fate specification. We contructed a strain defective in both *lin-17* and *lin-3*. 82% of *lin-17; lin-3* double mutants show P12 to P11 cell fate transformation, resembling *lin-44; lin-3* double mutants (P=0.544), while different from *lin-17* single mutants (P<0.0001). Therefore, similar synergistic interactions exist between *lin-3* and *lin-17* as between *lin-3* and *lin-44*. A synergistic interaction is also found between mutations of *let-23*, the EGF receptor for LIN-3 signal, and the Wnt signal LIN-44 (Table 2). Our data support the hypothesis that both of the two signaling pathways, *lin-3* and *lin-44*, are required for the P12 fate specification.

Table 2. Synergistic interactions between the lin-3/let-23 and the Wnt signaling pathways

Genotype	*n*	% P12→P11
*lin-3(n378/n1059)**	24	0
let-23(sy97)	50	44
lin-44(n1792)	244	16‡
lin-17(n671)	41	29§,¶
lin-17(n671) lin-44(n1792)	54	28§
lin-44(n1792): lin-3(n378/n1059)†	159	78‡
lin-17(n671); lin-3(n378/n1059)†	114	82¶
lin-44(n1792): let-23(sy97)	91	93**

*A strain of genotype + *let-312(s1234)lin-3(n378)* + *unc-22(s7)/unc-24(e138)+lin-3(n1059)dpy-20(e1282)* + was used to examine the P11/P12 phenotype at L3-L4 stage. Homozygotes of *lin-3(n1059)* are L1 lethal. Homozygotes of *lin-3(n378)* control display no P11/P12 defect.

†Full genotype of *lin-3* region is + *lin-3(n378) let-59(s49)* + *unc-22(s7)/unc-24(e138) lin-3(n1059)* + *dpy-20(e1282)* +. As homozygotes of either *lin-3(n1059)* or *let-59(s49)* are L1 lethal, only transheterozygous animals bearing *lin-3(n378/n1059)* would be viable therefore scored at L3-L4 stage.

‡$P<0.0001$, Fisher's Exact Test

§$P=1$, Fisher's Exact Test

¶$P<0.0001$, Fisher's Exact Test

**$P<0.0001$, Fisher's Exact Test, compared with additive effect which would be 53%.

Bi-directional P11/P12 cell fate transformation

During the course of our experiments, we found the surprising phenomenon that in two cases, overexpression of the same protein, EGL-5 or LIN-3, can have opposite effect on P11/P12 fate depending on the time of induction. Overexpression of EGL-5 induced in newly hatched larvae causes a P12 to P11 fate transformation (Fig. 2C), which contradicts its role in specifying P12 fate. Overexpression of LIN-3EGF during late embryogenesis potentiates the P12 to P11 cell fate transformation in a *lin-44(n1792)* background (Fig. 2B). *n1792* animals only exhibits 16% penetrance of the P12 to P11 fate transformation. When overexpression of LIN-3EGF is induced within several hours before hatching in a *lin-44* mutant background, the P12 to P11 fate transformation phenotype is increased to 70%. This bi-phasic profile does not occur when overexpression of LIN-3EGF is induced in a wild-type background (Fig. 2A) nor when *lin-44(n1792)* animals are treated with heat shock at the same stage (data not shown). This surprising phenomenon suggests to us two possibilities. First, the onset of *egl-5* expression might be crucial for proper P12 fate specification. Second, the unique effect of LIN-3 overexpression in *lin-44* mutant background suggests a requirement of *lin-44* function during late embryogenesis for P12 fate specification.

DISCUSSION

By studying the specification of the P11 and P12 cell fates in *C. elegans*, we address the question how a cell integrates multiple intrinsic and extrinsic factors to take on its correct fate. We considered three classes of genes: EGF signaling pathway genes, Wnt signaling pathway genes, and a HOM-C gene, which are involved in the P12 neuroectoblast fate specification. Our results support three conclusions. First, LIN-3 is an inductive signal for P12 fate and the *lin-3/let-23* EGF signaling pathway is required for P12 fate specification. Second, the HOM-C gene *egl-5* is a downstream target of the *lin-3/let-23* pathway in executing P12 fate. Third, the Wnt gene *lin-44* acts in parallel with the LIN-3 signal but at a different developmental time, and the Wnt pathway may regulate the competence of cells to respond to the LIN-3 inductive signal.

The conserved EGF signaling pathway is used to organize cell fate specification in the *C. elegans* tail region

Previous studies have indicated that mutations in several genes of the *lin-3/let-23* pathway displayed P11/P12 cell fate specification defects (Fixsen et al., 1985; Aroian and Sternberg, 1991; Clark et al., 1992b). The genes *let-23*, *lin-15*, *sem-5*, and *let-60* are all involved in P11/P12 cell fate specification. We provide evidence that LIN-3 is an inductive signal for P12 fate. First, overexpression of the EGF domain of LIN-3 leads to P11 to P12 cell fate transformation. Second, loss-of-function mutations of *lin-3* decrease P12 fate specification to the reverse transformation in a sensitized *lin-44* mutant background. This is the first demonstration that mutation of *lin-3* does have an effect on P12 fate specification, and therefore the effect of LIN-3EGF overexpression is not simply mimicking the effect of another EGF-like protein. Our

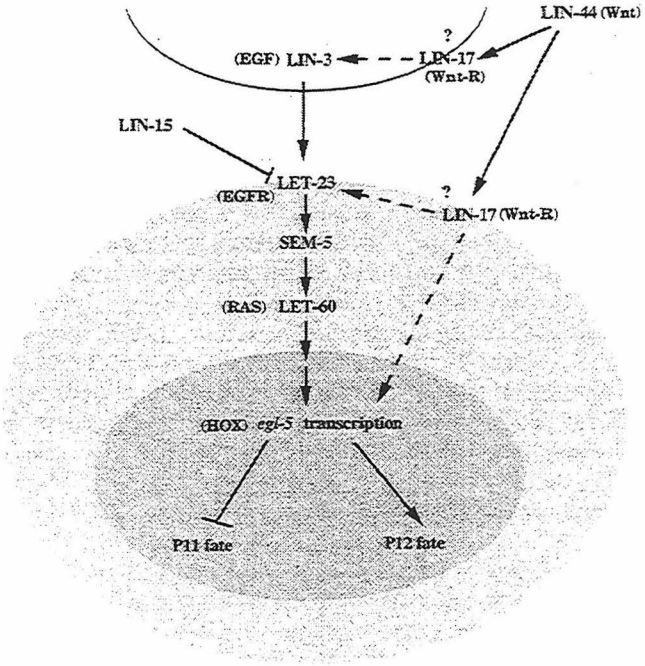


Fig. 4. Model for P12 fate specification. Inductive signal LIN-3 activates LET-23 receptor, which activates LET-60 RAS activity and turns on *egl-5* expression. Expression of *egl-5* at proper time specifies the cell to take on P12 fate. *lin-15* negatively regulates *let-23* activity and prevents the cell becoming P12. Wnt pathway may function at early developmental stage, LIN-44 signal acts via receptor LIN-17 to set up the competence of P11 and P12 cells to respond to the inductive signal and be able to express P12 fate. Integration of Wnt signal to the *lin-3/let-23* EGF signaling pathway may be at the level of LIN-3 signal, LET-23 receptor or *egl-5* transcription.

epistasis analysis suggests that the *lin-3/let-23* signaling pathway specifies P12 fate in the same fashion as it does for vulval induction. The same *lin-3/let-23* pathway has also been shown to organize *C. elegans* male spicule development (Chamberlin and Sternberg, 1994). Thus a conserved EGF signaling pathway is used at different times and in different cell types during development to organize cell fate specification.

During *C. elegans* hermaphrodite vulval development, a signal from the anchor cell (AC) in the gonad is required to induce three AC-proximal vulval precursor cells (VPC) to take on vulval fate, whereas the three distal VPCs produce nonspecialized epidermis (reviewed by Horvitz and Sternberg, 1991). This AC signal is an EGF-like protein encoded by the gene *lin-3* (Hill and Sternberg, 1992). *lin-3* expression revealed by a *lin-3-lacZ* reporter is detected in the AC at proper time for vulval induction (Hill and Sternberg, 1992). In male spicule development, *lin-3* mutants display defects similar to those observed in animals with F and U cells ablated (Chamberlin and Sternberg, 1993, 1994), suggesting that F and U cells may be the source of LIN-3 signal. The expression pattern of *lin-3* has not been precisely determined since the genomic clone used to determine AC expression does not contain the entire coding region (J. Liu, P. Tzou, R. Hill, and P. Sternberg, unpublished). What is the LIN-3 signal source in the hermaphrodite tail region for P12 fate specification? By analogy to vulva and spicule development, the LIN-3 signal source should be posterior to the P11 and P12 cells to promote the posterior fate, P12. Transgenic animals bearing a *lin-3-lacZ* reporter gene with additional genomic sequence have been generated (R. Hill, C. Chang and P. Sternberg, unpublished observation). Indeed we found that *lin-3-lacZ* is expressed in the hermaphrodite tail region in newly hatched larvae, in the cells K, K′, and the daughters of K, just posterior to the P11/P12 cells. To verify that the reporter gene expression truly reflects LIN-3 activity, we need to identify the source of the inductive signal for the P12 fate. Ablation experiments have been conducted to identify the source (Sulston and White, 1980; R. Hill, personal communication; L. Jiang, unpublished observation). We eliminated cells, including repD, K, K′, F and U, or combinations of these cells, in the rectal region by a laser microbeam to examine if the P12 fate would be disrupted. As the cells in the rectal region are essential for animal survival and growth, and the development of animals with ablated cells arrests before we could score the cell fates, such ablation attempts have been unsuccessful.

HOM-C gene *egl-5* is regulated by the EGF signaling pathway

HOM-C genes function in multiple cell types and at multiple times during development. Their expression patterns are finely tuned by diverse intercellular and intracellular interactions, which are important for the functions of HOM-C genes. *C. elegans* HOM-C genes, *lin-39*, *mab-5* and *egl-5*, are turned on early during embryogenesis in a position-specific manner from anterior to posterior along body axis (Wang et al., 1993). Their dynamic expression patterns and the regulation of their expression patterns have not been studied in detail until recently (Harris et al., 1996; Salser and Kenyon, 1996; Maloof and Kenyon, 1998). Here we have studied the interaction between the HOM-C gene, *egl-5*, and the *lin-3/let-23* EGF signaling pathway in the specification of P11/P12 cell fates and demonstrated that *egl-5* is a downstream target of the *lin-3/let-23* intercellular signaling pathway in specifying the P12 fate. *egl-5* expression is turned on in P12 lineage upon activation of the *lin-3/let-23* pathway, and it is necessary for P12 fate specification.

Interactions between HOM-C genes and the *lin-3/let-23* pathway have also been examined in *C. elegans* vulval development. Genetic analysis revealed two functions of *lin-39* during vulval development (Clark et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998): early LIN-39 activity specifies VPCs by creating a permissive condition for vulval differentiation; later LIN-39 activity regulates the response of VPCs to the *lin-3/let-23* pathway in the specification of a subset of VPCs as vulval tissue. Epistasis analysis has suggested that for the late role LIN-39 either regulates essential effectors of the response to inductive signal or itself is a target of the *lin-3/let-23* signaling pathway. Our molecular genetic analysis provides evidence that in the case of P12 fate specification, the HOM-C gene *egl-5* is a downstream target of the *lin-3/let-23* inductive pathway. If *lin-39* is indeed a target of the *lin-3/let-23* pathway during vulval development, as the data of Clandinin et al. (1997) and Maloof and Kenyon (1998) suggest, HOM-C genes may thus provide cell-specific responses to the same signaling pathway by being distinct targets.

lin-44 and *lin-3* act synergistically

Interactions between Wnt and growth signaling pathways can be either synergistic or antagonistic. Co-operative interactions between Wnt genes and members of the fibroblast growth factor (FGF) family have been identified during *Xenopus* embryonic development (Christian et al., 1992) and mammalian oncogenesis (Shackleford et al., 1993; Pan et al., 1995). Recently antagonistic interactions between *wingless* and EGFR signaling pathways have also been demonstrated during *Drosophila* larval cutical patterning (Szüts et al., 1997). Here we observed synergistic interactions between a Wnt protein, LIN-44, and the EGF-like protein LIN-3 in *C. elegans* P12 neuroectoblast fate specification. Moreover, this synergistic interaction also exists between the hypothesized Wnt receptor LIN-17 and LIN-3.

How do these two signaling pathways function in concert to specify P12 fate? We favor the model that both pathways are required for proper P12 fate specification and they act at different developmental times. First, LIN-3 overexpression experiments indicate that LIN-3 signal is required in early L1 before P11/P12 enter the ventral cord to induce P12 fate. Second, *lin-44* expression is turned on during embryogenesis, much earlier than the time of P11/P12 induction (Herman et al., 1995). Third, the unique effect of overexpression of LIN-3EGF during late embryogenesis in *lin-44* mutant suggests that *lin-44* function may be important in the early phase of P12 fate specification. It is possible that the Wnt pathway regulates the competence of the cells to respond to the LIN-3 inductive signal. But the Wnt signal alone is not sufficient to promote P12 fate, as overexpression of LIN-44 in wild-type animals has no effect on P11/P12 cell fate specification (L. Jiang and M. Herman, unpublished). These two pathways may also interact at multiple levels, for example, the Wnt pathway can regulate both the signaling cell and the responding cell. We set out to test the simple possibility that *lin-44* affects P12 fate by

regulating *lin-3* expression in the tail region. Results from three sets of experiments do not support this hypothesis. First, overexpression of LIN-3EGF can not completely bypass the requirement for *lin-44* function. A *lin-44* mutant decreased the response of P11 cell to LIN-3EGF overexpression (Fig. 2A,B). In addition, overexpression of LIN-3EGF during late embryogenesis caused a unique reverse cell fate transformation in a *lin-44* mutant background (Fig. 2B). Second, *lin-44* mutation did not affect *lin-3* expression as revealed by a *lin-3-lacZ* reporter gene. Third, decrease of *lin-3* activity had a more severe effect on P11/P12 fates than decrease of *lin-44* activity in a sensitized genetic background. Although our data do not favor this simple hypothesis, we can not completely rule out the possibility that *lin-44* somehow affects the property of the *lin-3* expressing cell in the tail region or the Wnt pathway may regulate LIN-3 protein processing. Further elucidation of how and where these two signaling pathways intersect requires additional genetic and molecular studies of both pathways.

Temporal regulation of gene interactions

Temporal control of gene activity is crucial for proper gene function during development. During our study of P12 neuroectoblast fate specification, we observed an unexpected phenomenon, in that overexpression of the same protein can result in opposite P11/P12 cell fate transformation depending on the time of induction. When overexpression of EGL-5 is induced in newly hatched larvae, it causes a P12 to P11 fate transformation (Fig. 2C), which contradicts its role in specifying P12 fate. This observation leads us to propose that the timing of *egl-5* expression is critical for P12 fate specification. To specify P12 fate, *egl-5* expression has to be turned on around the time of the first division of P11/P12 cells. If *egl-5* expression is turned on earlier, P12 fate would not be specified correctly. Consistent with this, in wild-type animals *egl-5-lacZ* expression is not detected in P12 cell at the early L1 stage (Wang et al., 1993). Similar ON-OFF behavior occurs in the activity of another HOM-C gene, *mab-5* (Salser and Kenyon, 1996). *mab-5* expression switching on and off in a lineage has been demonstrated to be important to control cell proliferation, differentiation and morphogenesis during development (Salser and Kenyon, 1996). In the case of LIN-3, when overexpression of LIN-3EGF is induced during late embryogenesis in a *lin-44(n1792)* background, it potentiates the P12 to P11 fate transformation caused by a *lin-44* mutation (Fig. 2B). This effect depends on both overexpression of LIN-3EGF and lack of LIN-44 activity. As activation of the *lin-3/let-23* pathway can turn on *egl-5* expression at the time of P12 fate induction, it might be that lack of LIN-44 activity enables *egl-5* expression to be turned on by LIN-3EGF earlier than usual, thus disrupting P12 fate specification. The timing difference in these two cases is consistent with this hypothesis as the effect of LIN-3 overexpression is earlier than that of EGL-5 overexpression. Another example of the bi-directional P11/P12 fate transformation is found in *mab-5* mutants (Kenyon, 1986). Loss-of-function mutants of *mab-5* show P11 to P12 as well as P12 to P11 fate transformations. As mutual repression has been suggested between HOM-C genes (Salser and Kenyon, 1993), temporal and spatial expression of *egl-5* might be abnormal in *mab-5* mutants, therefore resulting in cell fate transformation in both directions. A direct test of this hypothesis will require detailed temporal and spatial analysis of *egl-5* expression pattern in different genetic backgrounds.

Model for P12 fate specification

We propose the following model for P12 neuroectoblast fate specification (Fig. 4). In newly hatched larvae, LIN-44 signal acts via receptor LIN-17 to set up the competence of P11 and P12 cells to respond to the inductive signal and be able to express P12 fate. *egl-5* expression is kept off in both P11 and P12 cells. Later, an inductive signal LIN-3 coming from the posterior region activates LET-23 receptor activity in the posterior cell of the P11/P12 pair. Activation of the *lin-3/let-23* pathway turns on *egl-5* expression, which specifies the posterior cell to take on P12 fate. *lin-15* negatively regulates *let-23* activity and prevents the anterior cell becoming P12. Information from the Wnt signaling pathway may be integrated into the *lin-3/let-23* EGF signaling pathway at the level of LIN-3 signal, LET-23 receptor or *egl-5* transcription. Thus the temporal and spatial co-ordination and interactions between the Wnt signal, EGF signal and HOM-C transcription factor are important for P12 fate specification.

We are grateful to Craig Hunter for sending us the *egl-5* cDNA clone and the *muIs13* strain. We thank Michael Herman for the *lin-17(n671) lin-44(n1792)* strain and for many stimulating discussions. We thank Michael Herman, Howard Lipshitz, Kai Zinn, Tom Clandinin, Maureen Barr, Jing Liu, Minqin Wang, Peter Becker, Marie-Anne Félix, Giovanni Lesa, Aidyl Gonzalez-Serrichio, Chris Lacenere, René Garcia and Bhagwati Gupta for comments on the manuscript. This work was supported by a USPHS grant HD23690 to P. W. S. and by the HHMI, with which P. W. S. is an investigator.

REFERENCES

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

Aroian, R. V. and Sternberg, P. W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. *Genetics* **128**, 251-277.

Aroian, R. V., Lesa, G. M. and Sternberg, P. W. (1994). Mutations in the *Caenorhabditis elegans let-23* EGFR-like gene define elements important for cell-type specificity and function. *EMBO J.* **13**, 360-366.

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

Chamberlin, H. M. and Sternberg, P. W. (1993). Multiple cell interactions are required for fate specification during male spicule development in *Caenorhabditis elegans*. *Development* **118**, 297-324.

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

Chisholm, A. (1991). Control of cell fate in the tail region of *C. elegans* by the gene *egl-5*. *Genes Dev.* **111**, 921-932

Christian, J. L., Olson, D. J. and Moon, R. T. (1992). *Xwnt-8* modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* **11**, 33-41.

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

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992a). *C. elegans* cell-signaling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992b). Genes involved in two *Caenorhabditis elegans* cell-signaling pathways. *Cold Spring Harbor Symp. Quant. Biol.* **57**, 363-373.

Clark, S. G., Chisholm, A. D. and Horvitz, H. R. (1993). Control of cell

fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. *Cell* **74**. 43-55.

Clark, S. G., Lu, X. W. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes 2 different proteins. *Genetics* **137**. 987-997.

Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993). Signaling by receptor tyrosine kinases. *Ann. R. Bioch.* **62**. 453-481.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Genetics* **93**. 189-198.

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

Ferguson, E. L., Sternberg, P. W. and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**. 259-267.

Fixsen, W., Sternberg, P. W., Ellis, H. and Horvitz, H. R. (1985). Genes that affect cell fates during the development of *Caenorhabditis elegans*. *Cold Spring Harbor Symp. Quant. Biol.* **50**. 99-104.

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

Harris, J., Honigberg, L., Robinson, N. and Kenyon, C. J. (1996). Neuronal cell-migration in *Caenorhabditis elegans* – regulation of hox gene-expression and cell position. *Development* **122**. 3117-3131.

Herman, M. A. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. *Development* **120**. 1035-1047.

Herman, M. A., Vassilieva, L. L., Horvitz, H. R., Shaw, J. E. and Herman, R. K. (1995). The *C. elegans* gene *lin-44*, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. *Cell* **83**. 101-110.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in surrounding hypodermis. *Nature* **348**. 169-171.

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

Horvitz, H. R. and Sternberg, P. W. (1991). Multiple intercellular signaling systems control the development of the *Caenorhabditis elegans* vulva. *Nature* **351**. 535-541.

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

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

Immergluck, K., Lawrence, P.A. and Bienz, M. (1990). Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* **62**. 261-268

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

Kenyon, C. (1986). A gene involved in the development of the posterior body region of *Caenorhabditis elegans*. *Cell* **46**. 477-487.

Kornfeld, K. (1997). Vulval development in *Caenorhabditis elegans*. *Trends Genet.* **13**. 55-61.

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

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

Lesa, G. M. and Sternberg, P. W. (1997). Positive and negative tissue-specific signaling by a nematode epidermal growth factor receptor. *Mol. Biol. Cell* **8**. 779-793.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**. 565-570.

Maloof, J. N. and Kenyon, C. (1998). The hox gene *lin-39* is reuired during *C. elegans* vulval induction to select the outcome of Ras signaling. *Development* **125**. 181-190.

Mastick, G. S., Mckay, R., Oligino, T., Donovan, K. and Lopez, A. J. (1995). Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of *Ultrabithorax* protein-binding sites in yeast. *Genetics* **139**. 349-363.

Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C. elegans* – extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**. 3959-3970.

Moon, R. T., Brown, J. D. and Torres, M. (1997). Wnts modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**. 157-162.

Nusse, R. and Varmus, H. E. (1992). Wnt genes. *Cell* **69**. 1073-1087.

Pan, M. G., Wang, Y. H., Hirsch, D. D., Labudda, K. and Stork, P. J. (1995). The Wnt-1 proto-oncogene regulates MAP kinase activation by multiple growth factors in PC12 cells. *Oncogene* **11**. 2005-2012.

Russnak, R. H. and Candido, E. P. M. (1985). Locus encoding a family of small heat-shock genes in *Caenorhabditis elegans* – 2 genes duplicated to form a 3.8-kilobase inverted repeat. *Molec. Cell. Biol.* 5. 1268-1278.

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

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

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

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

Sawa, H., Lobel, L. and Horvitz, H. R. (1996). The *Caenorhabditis elegans* gene *lin-17*, which is required for certain asymmetric cell divisions, encodes a putative protein similar to seven-transmembrane protein similar to the *Drosophila* Frizzle protein. *Genes Dev.* **10**. 2189-2197.

Shackleford, G. M., Willert, K., Wang, J. W. and Varmus, H. E. (1993). The *Wnt-1* protooncogene induces changes in morphology, gene-expression, and growth-factor responsiveness in PC12 cells. *Neuron* **11**. 865-875.

Sternberg, P. W., Hill, R. J., Jongeward, G., Huang, L. S. and Carta, L. (1992). Intercellular signaling during *Caenorhabditis elegans* vulval induction. *Cold Spring Harbor Symp. Quant. Biol.* **57**. 353-362.

Sternberg, P. W. and Horvitz, H. R. (1988). *lin-17* mutations of *Caenorhabditis elegans* disrupt certain asymmetric cell divisions. *Dev. Biol.* **130**. 67-73.

Stringham, E. G., Dixon, D. K., Jones, D. and Candido, E. P. M. (1992). Temporal and spatial expression patterns of the small heat-shock *(hsp-16)* genes in transgenic *Caenorhabditis elegans*. *Molec. Biol. Cell* **3**. 221-233.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**. 110-156.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**. 577-597.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomson, J. N. (1983). The embryonic-cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**. 64-119.

Sundaram, M. and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. *BioEssays* **18**. 473-480.

Szüts, D., Freeman, M. and Bienz, M. (1997). Antagonism between EGFR and Wingless signaling in the larval cutical of *Drosophila*. *Development* **124**. 3209-3219.

van den Heuvel, M., Nusse, R., Johnson, P. and Lawrence, P. A. (1989). Distribution of wingless protein in *Drosophila* embryos: a protein involved in cell-cell communication. *Cell* **59**. 739-749.

Vincent, J. P. and Lawrence, P. A. (1994). Developmental genetics – it takes three to distalize. *Nature* **372**. 132-133.

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

Chapter III

A screen for mutants defective in neurogenesis during *C. elegans* male spicule development

ABSTRACT

To study terminal cell fate specification during *C. elegans* male spicule development, a molecular marker specific for the spicule neuron SPD fate is identified and characterized. Using this marker I examined the specification of the SPD fate in various mutant backgrounds. I also screened for new mutants that specifically disrupt marker expression in the spicule region. Results from these experiments suggest that the mechanism for SPD fate specification is different from that of other conserved neurogenic lineages. Multiple signaling pathways and cell-cell interactions must be involved in cell fate specification during *C. elegans* male spicule development.

INTRODUCTION

The specification of a certain cell fate during development can involve several mechanisms, including cell signaling events, local cell-cell interactions, and asymmetric cell divisions. We have been studying the mechanisms of cell fate specification during *C. elegans* male spicule development. *C. elegans* male spicules are generated by a single B cell, which is one of the four male specific blast cells (F, U, B, and Y) that divide postembryonically and produce some of the specialized mating structures in the male tail (Sulston and Horvitz, 1977, Sulston et al., 1980). The mechanisms of B lineage cell fate specification at early stage (1-cell stage to 10-cell stage, L1-eL3) have been studied intensively recently (Chamberlin and Sternberg, 1993, 1994, 1995). Both asymmetric cell divisions and cell-cell interactions are involved in this process. Signaling pathways involved in this process include the Wnt, EGF, and Notch pathways.

The B lineage generates 10 progeny by the end of L2 stage. After the cell divisions, the cells undergo short range migration to assume their correct positions. During the L3 stage, these 10 cells continue to divide to generate spicule neurons, sheath cells, socket cells, and connective tissues. With the exception of two socket cells generated by α and γ, the spicule neurons and structural cells are mainly derived from the β and ζ sublineages (Figure 1C), whereas other sublineages give rise to part of the proctodeum which is involved in spicule morphogenesis to ensure the correct connection between spicules and other structures in the tail region (i.e., genital tract, sex-specific muscles, etc.). The β and ζ sublineages are particularly interesting, not only

because they generate most of the spicule cells, but also they are neurogenic lineages. The cell division pattern of the neuorgenic lineages are evolutionary conserved both in *C. elegans* and in other organisms (e.g., *C. elegans* male sensory ray lineage, *Drosophila* bristle lineage, etc.). Development of the *Drosophila* bristle lineage has been extensively studied and is best understood. Both intrinsic and extrinsic mechanisms are used to specify cell fates in the *Drosophila* bristle lineage. The sensory organ precursor (SOP) cell fate is determined via inhibitory cell-cell interactions mediated by the *Notch/Delta* pathway (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). The SOP cell divides twice to generate four distinct cell fates. Each cell division is asymmetric, producing two daughters with distinct cell fates. Asymmetric segregation of the NUMB and PROSPERO proteins provides intrinsic cues for these asymmetric divisions (Rhyu et al., 1994; Hirata et al., 1995). The *Notch/Delta* pathway mediates interactions between the two daughter cells. It remains to be determined how neuronal vs. non-neuronal cell fates are specified in the β and ζ lineages during *C. elegans* male spicule development, if similar mechanisms are utilized, or if other mechanisms are involved.

In *C. elegans*, the conventional method for studying cell fate specification is based on cell division pattern and cell anatomy (e.g., Chamberlin and Sternberg, 1993). Yet this method can not detect subtle lineage defect or execution of terminal cell fate. Therefore, identification of a molecular marker that is expressed specifically in some of the terminal cell fates is crucial for this study. Here I report the identification and characterization of a SPD neuron specific marker, *gpa-1::lacZ*. Using this

marker I examined specification of the SPD fate in a variety of mutant backgrounds. I also performed a genetic screen looking for mutants that disrupt marker gene expression specifically in the spicule region. These studies provide further insights on the development of ζ lineage and SPD fate specification.

MATERIAL AND METHODS

General culture

Routine culturing, maintenance and genetic manipulations of *C. elegans* strains were performed according to standard procedures (Brenner, 1974). N2 (Bristol) strain was used as wild type (Brenner, 1974). Standard cellular and genetic nomenclature is as defined by Sulston and Horvitz (1977) and Horvitz et al. (1979). The following strains were used.

LGI: *unc-13(e51), dpy-5(e61), cod-7(sy190)*

LGII: *unc-4(e120), dpy-10(e128), lin-31(n301), lin-31(bx31), unc-86(e1416), sy579*

LGIII: *unc-32(e189), dpy-17(e164), dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909), lin-48(sy234), lin-48(sy548), egl-5(n945), egl-5(sy546), pha-1(e2123ts), cod-4(sy180), cod-6(sy186), sy549*

LGIV: *lin-3(n378) let-59(s49) unc-22(s7)/unc-24(e138) lin-3(n1059) dpy-20(e1282), dpy-20(e1282), dpy-20(e1362), dpy-20(e1282) unc-24(e138), dpy-13(e184), let-60(sy103gf), lin-1(e1777), cod-3(sy166)*

LGV: *unc-42(e270), syIs20 him-5(e1490), dpy-11(e224), lin-32(u282), cod-10(sy193)*

LGX: *unc-3(e151), lon-2(e678)*

Linkage not known: *sy158, 6T6, 8T9*

PS1701: *dpy-20(e1362); syIs19[gpa-1::lacZ+dpy-20(+)] him-5(e1490)*

PS1702: *dpy-20(e1362); syIs20[gpa-1::lacZ+dpy-20(+)] him-5(e1490)*

PS2255: *dpy-20(e1282); him-5(e1490); syIs33[dpy-20(+)+gpa-1::GFP(50ng / μl)]*

PS2471: *pha-1(ts); him-5(e1490); syEx181[pha-1(+)+sra-1::GFP (S65C) (50ng / μl)]*

PS2648: *syIs6[hs-lin-3EGF+unc-31(+)]; syIs19[gpa-1::lacZ+dpy-20(+)] him-5(e1490)*

PS2676: *pha-1(e2123ts); dpy-20(e1282); syIs20 him-5(e1490); syEx220 [JK51(hs-GLP-1ANK) 20ng / μl + pha-1(+)]*

EMS mutagenesis

The strain *syIs20 him-5(e1490)* was used for the F1 clonal screen. Standard EMS mutagenesis was performed. 5-10 F1 hermaphrodites were picked individually from each P0 plate. At F2 generation, 10-20 adult males from each F1 plate were picked to 96-well microtiter plates, stained for β-galactosidase activity, and the *gpa-1::lacZ* marker expression patterns were examined. Expression patterns were pre-screened under dissecting microscope. If abnormal expression pattern was observed, worms were then mounted onto 5% agar pad slides and examined in detail under Nomarski optics. If ~1/4 of males showed specific abnormalities, F2 hermaphrodites were picked individually from the corresponding plate and F3 males were examined to verify the phenotype and to screen for homozygotes.

β-galactosidase activity

Assay for β-galactosidase activity was performed as described previously (Fire, 1992). A 96-cell microtiter plate was used for analyzing many different strains simultaneously.

Genetic Mapping

Mapping of PS1702

PS1701 and PS1702 are X-ray integrated lines with the *gpa-1::lacZ* reporter construct and the transformation marker pMH86 (dpy-20(+)) inserted onto chromosomes (J. Mendel, personal communication). Insertion (Is) rescues *dpy-20* phenotype. Transgenic males are crossed into *dpy-20; unc* strains, cross progeny of non-Dpy non-Unc hermaphrodites were picked. At F2 generation Unc-non-Dpy hermaphrodites were picked individually and *dpy-20* segregation was scored at F3 generation. If *Is* is linked to *unc*, Unc-non-Dpy would be rare and all Unc-non-Dpy should segregate ~1/4 *dpy-20* progeny; if *Is* is not linked to *unc*, ~1/3 Unc-non-Dpy would not segregate *dpy-20* and ~2/3 Unc-non-Dpy would segregate ~1/4 *dpy-20* progeny.

PS1702:

I: 5/6 ~1/4 D; 1/6 nD; II: 4/8 ~1/4 D; 4/8 nD;

III: 5/8 ~1/4 D; 3/8 nD; IV: 6/8 ~1/4 D; 2/8 nD;

V: only 1 Unc-non-Dpy F2 recovered, ~1/4 D

X: *Is* males are crossed into N2 hermaphrodites, all F1 males are *lacZ* staining positive. So it is not on X.

Mapping of mutants

Mapping strains with *dpy* or *lon* markers in *syIs20 him-5(e1490)* background were generated. Usually males from marker strains are mated with mutant hermaphrodites. Cross progeny were picked singly. At F2 generations *dpy* or *lon* males were tested for *gpa-1::lacZ* marker expression. If a mutant is not linked to the *dpy* or *lon* marker, 1/4 of males tested will display mutant phenotype. If a mutant is linked to the marker, mutant phenotype will be rarely seen in males tested.

Anatomical and lineage analysis / Cell ablation / Photographs

Animal anatomy and cell division patterns were examined under Nomarski optics at 20°C as described by Sulston and Horvitz (1977). For laser ablation, worms were mounted on 5% agar pad and anesthetized with 1-3% sodium azide. Laser killing of cells was performed as described (Sulston and White, 1980; Avery and Horvitz, 1987, 1989). Photographs of β-galactosidase expression patterns were taken on Kodak Royal Gold ASA 25 films.

RESULTS

Summary of spicule structure, function and development

C. elegans male spicules are a pair of sensory structures that are required for male copulatory behaviors. Each spicule is composed of two sensory neurons, two sheath cells and four socket cells (Sulston et al., 1980). The two neurons, SPD and SPV, send their dendrites through the spicule and are open to the environment via a hole at the tip of the spicule. The structural cells, sheath and socket, enwrap the neurons and anchor the

spicule to its retractor and protractor muscles. A third neuron, SPC, sends its dendrite to the base of the spicule and mediates spicule movement. Each of these neurons, sheath cells and socket cells has distinct and important role for spicule morphology and function. During mating, male locates hermaphrodite vulva, inserts its spicules and transfers sperms. The SPC and SPD neurons mediate spicule insertion, and the SPV neuron mediates sperm transfer (Liu and Sternberg, 1995). The sheath cells, which are glia-like cells, facilitate neuronal function. The socket cells are important for spicule morphogenesis (Chapter V).

The whole spicule structure is derived from a single male specific B blast cell. The B cell divides postembryonically. The first division of the B cell is asymmetric, generating a larger anterior-dorsal daughter B.a and a smaller posterior-ventral daughter B.p. The asymmetry of this division is controlled by the Wnt pathway genes *lin-44* and *lin-17* (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994). LIN-44 is a member of the Wnt protein family (Herman et al., 1995). LIN-17 is a homologue of the *Drosophila* FRIZZLED protein and has been implicated as the receptor for the Wnt signal LIN-44 (Sawa et al., 1996). Epistasis analyses suggest that LIN-44 act via LIN-17 to promote the posterior B.p fate (Chamberlin and Sternberg, 1995). VAB-3 is a Pax-6 class transcription factor, which is required for the anterior B.a fate and acts epistatically to *lin-17* (Chisholm and Horvitz, 1995; Chamberlin and Sternberg, 1995).

B.a generates all the cells of the spicules. By the end of L2 stage, B.a divides three times to form a ring of eight cells. B.p divides once generating two cells. A network of cell-cell interactions occurs and leads to the

specification of six distinct precursor cell types at 10-cell stage, designated as α, β, γ, δ, ε (2), and ζ (2) (Figure 1A, 1B). A signal mediated by the F and U cells is required to promote the anterior fates (α, γ, ε) in general (Chamberlin and Sternberg, 1993). Ablation of F and U cells results in anterior to posterior fate transformation. This signaling event is likely to be controlled by the *lin-3/let-23* EGF signaling pathway (Chamberlin and Sternberg, 1994). First, the B lineage defects caused by loss of function mutants in genes of the *lin-3/let-23* pathway, i.e., *lin-3, let-23, sem-5, let-60*, and *lin-45*, resemble those seen in F and U ablated males. Second, overexpression of LIN-3-EGF can partially compensate for the lack of F and U cells and lead to posterior to anterior fates transformation. The posterior fates (β, δ, ζ) are promoted by a signal from the Y.p cell and its progeny. When Y.p cell is ablated, some of the posterior cell lineages are abnormal (Chamberlin and Sternberg, 1993). *lin-12*, the *C. elegans* Notch homologue, is involved in the interaction between γ and δ fates (Chamberlin and Sternberg, 1994).

During the L3 stage, these cells continue to divide to generate spicule neurons, structural cells and connection tissues. The β and ζ sublineages are of particular interest as all the spicule neurons and most of spicule structural cells are generated by these two sublineages (Figure 1C). These sublineages are similar in that one cell divides twice to generate neuron, sheath cell, and socket cell. This type of lineage is also used in generating other neuronal structures during *C. elegans* development and in generating sensory organs of *Drosophila* (Figure 1C). During the development of *Drosophila* sensory organ precursor lineage (SOP), it has been shown that both intercellular

signaling pathway, Notch/Delta, and intracellular molecules, Numb, are used to specify the neuronal vs. non-neuronal terminal cell fates. For neurogenesis during *C. elegans* male spicule development, two questions arise: first, are similar pathways used in this conserved lineage to specify cell fates? second, how are the terminal cell fates specified?

Characterization of a molecular marker specific for the SPD neuronal fate

To study terminal cell fate specification, I needed to make use of molecular markers that are specifically expressed in certain terminal cell fates. *gpa-1::lacZ* is the first marker of this kind we identified. *gpa-1* encodes a G protein α subunit homologue (Lochrie et al., 1991). Transgenic animals bearing a *lacZ* reporter gene driven by *gpa-1* promoter is found to be expressed in male spicules as well as phasmid neurons in the tail and several cells in the head (J. Mendel, personal communication). In wild-type males, *gpa-1::lacZ* is found to be expressed in one cell per spicule. The blue cell sends processes through the spicule and to the ventral cord, suggesting it may be a spicule neuron. To determine exactly which cell expresses this marker, I performed laser ablation experiment with two independently integrated transgenic lines, PS1701 and PS1702. Using a laser microbeam I ablated certain cell or cell precursor during L3 stage and examined β-galactosidase expression in adult males. As shown in Figure 2 and Table 1, when both β and ζ are ablated, there is no marker expression in the spicules but the expression in phasmid neurons and in the head region remains.

When ζ is ablated, the spicule expression is eliminated yet β ablation does not affect spicule expression. This suggests that the spicule expression is from ζ lineage. Furthermore, when SPD neuron is ablated specifically, there is no expression in the spicule region. Therefore *gpa-1::lacZ* is a marker for SPD neuron. This result is obtained from both integrated lines (Table 1). *gpa-1::lacZ* expression in SPD neurons is turned on during L4 molt and stays on through adulthood. I further backcrossed the strain PS1702 and mapped it on chromosome V that is linked to the *him-5* mutation. Mutation in the *him-5* gene results in high ratio of male progeny at ~30% rather than wild-type 0.2%. Therefore the linkage of *gpa-1::lacZ* marker to *him-5* makes it genetically easier to generate male strains and examine male spicule expression. I have been using the PS1702 strain for my work thereafter.

Cell ablations

Using cell lineage analysis, Helen Chamberlin was able to show that multiple cell interactions between F, U, and B cells as well as between B progeny are required for fate specification during spicule development (Chamberlin and Sternberg, 1993). With the terminal cell fate marker I would be able to examine the effects of cell interactions on cell fate specification not only at the level of cell division patterns but also at the level of cell fate execution. F and U cells promote anterior fates in general. When F and U cells are ablated, the anterior α cell divides in a pattern resembling β lineage; γ and ε divide abnormally partial resembling posterior δ and ζ lineages, respectively; while all the posterior lineages, β, δ, and ζ appear to be

normal. When I ablated both F and U at the B lineage 2-cell stage, operated males displayed severely crumpled spicules and excessive marker expression in the spicule region (Figure 3A, Table 2). This would suggest that some of the anterior lineages, possibly ε, are at least partially transformed to ζ lineage. When the Fl/r and Ul/r cells are ablated at the B lineage 10-cell stage, males have wild-type spicules with wild-type marker expression pattern (Figure 3B, Table 2). Therefore signaling events between F, U cells and B cells must happen prior to 10-cell stage. Y.p cell is thought to promote posterior fates in general. When Y.p cell is ablated at B lineage 2-cell stage, crumpled spicules are generated but SPD marker expression appears to be wild-type (Figure 3C, Table 2). This observation is also consistent with cell lineage analysis that ζ lineage is not affected much in Y.p ablated animals.

I further examined cell interactions between B progeny. When the presumptive ζ pair is isolated at early 10-cell stage (at the time around the division of B.p cell) by ablating all other B.a progeny, no spicule is formed and no marker expression is observed in the spicule region (Figure 3D). However, if the presumptive ζ pair is isolated at late 10-cell stage when the B.a progeny have migrated to their positions, two cells expressing the *gpa-1::lacZ* marker are found in the spicule region although no spicule is formed (Figure 3E).

This set of ablation experiments suggests that the cell signaling events between F, U, and B cells happen prior to 10-cell stage, which is consistent with the results obtained by cell lineage analysis (Chamberlin and Sternberg, 1993). After 10-cell stage, those signals are not necessary for ζ fate

specification. Furthermore, ζ does not seem to require much interactions from its siblings to execute its fate once it acquires its correct position at 10-cell stage.

Screen of known mutants

lin-3/let-23 pathway mutants

The *lin-3/let-23* EGF signaling pathway has been implicated in cell fate specification at 10-cell stage as revealed by lineage analyses (Chamberlin and Sternberg, 1994). Mutants of the *lin-3/let-23* pathway components display lineage abnormalities after 10-cell stage, most commonly in α and β sublineages. To examine if the *lin-3/let-23* signaling pathway is involved in late B lineage development, I crossed the *gpa-1::lacZ* marker into various mutant backgrounds, including *hs-LIN-3EGF, lin-3(lf) let-60(gf), lin-31(lf),* and *lin-1(lf)*. Although some of the mutants have crumpled spicules, none of them display abnormal expression pattern of the *gpa-1::lacZ* marker in the spicule region (for each strain examined, n>20; Figure 4A, 4B).

lin-12

Notch/Delta pathway controls the neuronal vs. non-neuronal cell fate specification in both central nervous system and peripheral nervous system during *Drosophila* embryonic development (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). During the generation of neuroblast cells, the Notch/Delta pathway mediates interactions between epidermal cells and neuronal precursor cells, activation of this pathway leads to epidermal fate. During the development of *Drosophila* SOP lineage, Notch/Delta pathway mediates interactions between two daughter cells to ensure one adopt

neuronal fate, the other one non-neuronal fate. In male spicule development, the *C. elegans* Notch homologue *lin-12* has been implicated to mediate anterior vs. posterior fates at 10-cell stage, especially for the γ and δ pair (Yochem et al., 1988; Chamberlin and Sternberg, 1994). Activation of *lin-12* promotes posterior fate, inactivation of *lin-12* leads to posterior to anterior fate transformation. I wanted to test if *lin-12* plays a role for the neuronal vs. non-neuronal fate specification after 10-cell stage. By analogy to the *Drosophila* SOP lineage, I should expect that loss-of-function *lin-12* will lead to extra expression of the neuronal marker in the spicules, and gain-of-function *lin-12* will lead to loss of neuronal marker expression in the spicules. *gpa-1::lacZ* marker was crossed into *lin-12(o)/lin-12(d)* strain, *lin-12(o)* and *lin-12(d)* homozygous adult males from heterozygous mothers were examined for marker expression in spicules. Although variable crumpled spicules are found, the marker expression does not seem to be affected in *lin-12(o)* males. *lin-12(d)* males show mainly wild-type expression pattern with occasional loss of marker expression in the spicules (Figure 4C). Since *lin-12* may have two roles during spicule development, an early role and a late role, we chose to use a transgenic line PS2676 to activate the *lin-12* pathway at certain developmental point and test its effect. This transgenic line carries a construct, which contains the cytoplasmic ankyrin repeat region of GLP-1 driven by a heat shock promoter. This construct has been proven to behave like activated *lin-12* in many developmental processes (Roehl and Kimble, 1993). L3 worms were staged under Nomarski optics before heat shock. Phenotypes caused by *lin-12* activation, such as ectopic hook, ray fusion,

crumpled spicules, were observed after heat shock, yet the *gpa-1::lacZ* marker expression appeared to be normal (n>50).

lin-32/unc-86

lin-32 and *unc-86* are both involved in the generation of neuroblast cells in *C. elegans*. *lin-32* encodes a bHLH *achaete/scute* family transcription factor required for generation of the posterid and ray neuron precursors (Zhao and Emmons, 1995). *unc-86* encodes a POU domain transcription factor that is required for generation of touch receptor neurons (Finney et al., 1988). Moreover, *unc-86* is asymmetrically expressed during several cell divisions and this asymmetric expression pattern is coupled with lineage asymmetry (Baumeister et al., 1996). It is possible that such kind of factors may also control asymmetric cell division and cell fate specification in the generation of spicule neurons. To test this possibility, I crossed the SPD neuron marker to *lin-32* and *unc-86* mutant backgrounds and examined marker expression pattern. Loss-of-function *lin-32* mutant males although have no rays do have wild-type spicules, and SPD neurons seem to be correctly specified. In *lin-32* mutant males, *gpa-1::lacZ* expression is observed in SPD cell bodies as well as dendrites through the spicules and axons in the ventral cord (n>20; Figure 4D). *unc-86* mutation does not affect marker expression, either (n>20; Figure 4E).

cod

cod(copulation defective) mutants are copulatory defective mutants that have been isolated by Katherine Liu and Yvonne Hajdu-Cronin in the lab. Spicule insertion is one of the several steps of male mating behavior and is thought to be mediated mainly by SPD neurons. It would be interesting to see if any of

those spicule insertion defective mutants is missing SPD neurons by using the SPD marker. Using *rol-6*^{D} dominant marker for transformation selection I co-injected *gpa-1::lacZ* construct and *rol-6* DNA into spicule insertion defective mutants. Stable lines were generated and roller males were picked to examine β-galactosidase expression pattern. All of the spicule insertion defective mutants examined showed positive staining for SPD neurons (including *sy158, sy166, sy180, sy186, sy190, sy193*, at least two stable lines were examined for each mutant). It is possible that in those mutants the SPD neurons are specified but are not functional. Mating behavior is a complex behavior that requires concise coordination of multiple muscles and neurons. Therefore it is very likely that those spicule insertion defective mutants have defects other than neuronal fate specification.

Screen for new mutants

To discover new mutants that affect terminal cell fate specification during male spicule development, I performed an F1 clonal screen looking for mutants that disrupt the *gpa-1::lacZ* marker expression specifically in the spicule region (Figure 5). Since *gpa-1::lacZ* is also expressed in phasmid neurons and several cells in the head region, it provides an internal control to discard any general regulators for transcription. Two classes of mutants can be discovered from this screen. One class (I) would have early B lineage defect, and thus would have crumpled spicules and abnormal expression pattern. The other class (II) would have late B lineage defect, thus would have fairly normal spicule morphology but abnormal marker expression in

the spicules. From a screen of 6,354 haploid genome, I recovered four class I mutants and three class II mutants.

2D2

The *2D2* mutant has severely crumpled spicules with occasionally excessive marker expression in the spicule region (Figure 6A, 6B). About 10% males show an early B lineage defect (n=21). By L3 stage, the B cell has only divided twice to generate four progeny, while in wild-type animals B lineage should have developed to 10-cell stage. Those males do not form spicules and show no marker expression in the spicule region. Most mutant males appear to have an abnormal Y.p lineage. In wild-type animals, Y.p divides at L2 stage to generate 5 progeny. While in mutant males Y.p only generates 4 progeny. B lineage develops normally until 10-cell stage in most mutant males. After 10-cell stage several sublineages show various defects and result in severely crumpled spicules in adulthood. This mutant strain also shows a vulvaless phenotype in hermaphrodites. Based on the crumpled spicule phenotype, *2D2* maps to chromosome IV.

egl-5(sy546)

Males of this mutant strain appear to have general tail patterning defect. They do not have spicules or rays. They have a gonad connection defect. However, several cells that express the *gpa-1::lacZ* marker are observed in the spicule region. Cell lineage analysis reveals an early B lineage defect. At the L3 stage, B.a only has 4 progeny instead of wild-type 8, and B.p appears to divide normally. It is not clear how the marker expressing cells are generated. *sy546* is mapped to chromosome III and fails to complement a putative null allele of *egl-5*, *n945*. *egl-5* encodes a homologue of *Drosophila*

hox gene *Abdominal-B* and is known to play a role in general tail patterning (Chisholm, 1989; Wang et al., 1993). *sy546* is likely to be a hypomorphic allele of *egl-5*: *sy546* hermaphrodites are only slightly egg-laying defective (Egl-) and the P12 fate specification appears to be normal (n=20) while *n945* hermaphrodites are highly penetrant Egl- and show 100% penetrance P12 to P11 fate transformation (Chisholm, 1989).

lin-48(sy548)

sy548 was originally discovered due to the loss of *gpa-1::lacZ* marker expression in the spicule region. When examined under Nomarski optics, mutant males have crumpled spicules to various degree as well as some ectopic spicule material (Figure 6C), which is an indication of early lineage defects. I examined cell positions and cell morphology at the B lineage 10-cell stage and found that the F and U cells are abnormal in all animals examined (n=20). F, U, Fl/r, Ul/r are abnormally large and display extra cell divisions. The ectopic spicule material is deposited by ectopically formed socket cells generated by the abnormal U cell (Chapter V). The B lineage is also defective. At 10-cell stage, α and β cells are positioned left and right instead of anterior and posterior on the central focal plane. In some animals α lineage displays a similar division pattern as β in that α and αl/r divide at the same time as β and βl/r respectively, and αd divides an extra round as βd. However, the ζ lineage, which is the progenitor of SPD neuron, does not exhibit any cell division defect (n=9). Based on the abnormal F and U lineages, it resembles the *lin-48(sy234)* mutant, which is previously isolated from a spicule morphology screen (Chamberlin, 1994). Complementation test indicates that

sy548 fails to complement *lin-48(sy234)*. Therefore *sy548* is an allele of *lin-48*. The phenotype of loss of SPD marker expression is about 50% penetrant in both alleles. *lin-48* locus was cloned by Helen Chamberlin and it defines an Ovo-like transcription factor with four C2H2 zinc fingers (H. M. Chamberlin, personal communication). GFP reporter construct driven by *lin-48* promoter reveals that *lin-48* is expressed in F and U cells as well as several cells in the B lineage during L4 stage. The late expression of *lin-48* may indicate the involvement of *lin-48* in late stage B lineage cell fate specification as suggested by the *gpa-1::lacZ* marker.

***sy579* (a.k.a. *5U6*)**

The mutant *sy579* was recovered based on the phenotype of loss of marker expression in the spicule region (Figure 6D). Mutant males display B lineage defect after 10-cell stage and have crumpled spicules. They also have ray defects. At plate level, mutants look uncoordinated. I mapped to *sy579* chromosome II.

6T6

This mutant displays weaker marker expression in the male spicule region. When examined under Nomarski optics, the spicule cuticle at the spicule-muscle connection region looks abnormal (Figure 6E). Males have wild-type B lineage and otherwise wild-type spicules, but they can not mate when tested with Dpy hermaphrodites.

8T9

8T9 mutant males have wild-type spicules and wild-type tail but the *gpa-1::lacZ* marker expression is lost specifically in the spicule region (Figure 6F; *gpa-1::lacZ* expression in phasmid neurons is present). Cell lineage analysis

revealed no B lineage abnormality (n=3). Mutant males can mate with Dpy hermaphrodites at low efficiency. The elongated spicules and wild-type spicule cuticle suggests that the spicule socket cells are functional (see Chapter V). Thus the loss of SPD marker expression phenotype may be an indication of neuron to sheath fate transformation. Other terminal cell fate specific markers, especially markers for sheath cell fate, would be useful to further elucidate the mutant phenotype.

sy549

This mutant shows ectopic marker expression in the spicule region. In wild-type animals, *gpa-1::lacZ* marker is expressed in one cell per spicule. In *sy549* mutant this marker is expressed in two-cell per spicule (Figure 6G). Spicule morphology appears to be normal in mutant males. I have focused my effort on this mutant and the genetic and molecular characterization of this mutant will be discussed in detail in Chapter IV. The genetic mapping data for this mutant is summarized below.

sy549 mapping data:

Two-factor:

II: with *dpy-10*:

4/19 *dpy-10* males picked up *sy549*; 4/42 non-dpy males picked up *sy549*;

2/4 *dpy-10* hermaphrodites segregated *sy549*

III: with *dpy-17*:

0/32 *dpy-17* males picked up *sy549*; 5/40 non-dpy males picked up *sy549*;

0/5 *dpy-17* hermaphrodites segregated *sy549*

V: with *dpy-11*:

5/19 *dpy-11* males picked up *sy549*; 5/35 non-dpy males picked up *sy549*;

3/6 *dpy-11* hermaphrodites segregated *sy549*

Three-factor:

dpy-1 unc-32:	14/21 Dpy-non-Unc picked up *sy549*
	2/27 Unc-non-Dpy picked up *sy549*
unc-32 dpy-18:	0/ Unc-non-Dpy picked up *sy549*
	11/12 Dpy-non-Unc picked up *sy549*
dpy-18 unc-25:	4/4 Unc-non-Dpy picked up *sy549*
	5/15 Dpy-non-Unc picked up *sy549*
dpy-17 unc-49:	7/18 Dpy-non-Unc picked up *sy549*
	8/13 Unc-non-Dpy picked up *sy549*
unc-93 dpy-17:	8/12 Unc-non-Dpy picked up *sy549*
	1/9 Dpy-non-Unc picked up *sy549*
dpy-17 unc-32:	1/6 Unc-non-Dpy picked up *sy549*
	1/3 Dpy-non-Unc picked up *sy549*
dpy-17 unc-36:	5/9 Unc-non-Dpy picked up *sy549*
	4/13 Dpy-non-Unc picked up *sy549*
dpy-17 unc-86:	2/7 Unc-non-Dpy picked up *sy549*
	4/11 Dpy-non-Unc picked up *sy549*
dpy-17 lon-1:	0/2 Lon-non-Dpy picked up *sy549*
lon-1 unc-32:	7/21 Unc-non-Lon picked up *sy549*
	12/16 Lon-non-Unc picked up *sy549*

Deficiency mapping:

sDf125: dpy-17 sy549 / dpy-17 sDf125 unc-32

males: 18/23 *dpy-17* males showed 2-cell/spicule expressing marker

2/27 wild-type siblings showed 2-cell/spicule expressing marker

hermaphrodites: 17/18 were sterile or had bursting vulva

1/18 brood size less than 10

sDf127: dpy-17 sy549 / dpy-17 sDf127 unc-32

males: 24/30 *dpy-17* males showed 2-cell/spicule expressing marker

3/16 wild-type siblings showed 2-cell/spicule expressing marker

hermaphrodites: 17/23 were sterile or had bursting vulva

6/23 brood size less than 10

sDf135: dpy-17 sy549 / dpy-17 sDf135 unc-32

males: 7/7 *dpy-17* males showed wild-type marker expression

15/18 wild-type siblings had wild-type marker expression

nDf16: sy549 unc-32 / nDf16, 11/15 showed wild-type expression

dpy-17 unc-32 / nDf16, 8/10 showed wild-type expression

nDf20: sy549 unc-32 / nDf20, 9/10 showed wild-type expression

DISCUSSION

ζ fate specification in male spicule development

Using a molecular marker that is specifically expressed in one of the terminal cell fates, I was able to study the terminal cell fate specification during spicule development. The *gpa-1::lacZ* marker is specifically expressed in SPD neurons, which is derived from the ζ lineages. Studies by Chamberlin and Sternberg (1993, 1994) suggest that the ζ lineage is hard to perturb or duplicate. F and U cells are thought to promote the anterior fates (α, ε, γ) in general. Ablation of F and U cells results in anterior fates to posterior fates

(β, ζ, δ) transformation (Chamberlin and Sternberg, 1993). But the ε lineage, the anterior equivalence of ζ, does not transform to ζ lineage completely. Rather it displays an intermediate or hybrid lineage. With the *gpa-1::lacZ* marker I observed excessive marker expression in the spicule region when F and U cells are ablated at the B lineage 2-cell stage. This result together with lineage analysis data suggests that ε lineage is at least partially transformed to ζ lineage and ε progeny is able to express the SPD fate which is normally derived from ζ lineage. Therefore, signals from F and U cells indeed promote the anterior fates in general. Ablation of Y.p, the signal source for some of the posterior fates, does not affect marker expression in the spicule region. This observation is consistent with the cell lineage analysis (Chamberlin and Sternberg, 1993).

Interactions between B.a progeny do play a role for ζ fate specification prior to 10-cell stage. When ζ pair is isolated by removing all other B.a progeny at 9-cell stage or early 10-cell stage, the SPD fate fails to be expressed as no marker expression is observed. Lineage analysis revealed that ζ fate is transformed to ε fate (Chamberlin and Sternberg, 1993). However, if ζ pair is isolated two hours later when B.a progeny have finished their short range migration and take on their proper positions, SPD fate can be expressed correctly. Thus the cell-cell interactions between B.a progeny must occur around the 10-cell stage during the period of short range migration of these cells. Once ζ assumes its correct position, it does not require further interactions from B.a progeny to execute its lineage and

terminal cell fates. However, this does not exclude signaling events from other neighboring cells (Chapter IV).

Spicule neurogenesis

The SPD neuron specific marker provides a chance to study neurogenesis during *C. elegans* male spicule development. Using this marker I examined a variety of known mutants, including the *lin-3/let-23* EGF signaling pathway, the *C. elegans Notch* homologue *lin-12*, genes involved in neuroblast generation, and several *cod* mutants. Surprisingly none of these mutants display any defect on SPD fate specification. The *Notch/Delta* signaling pathway plays important roles in the neurogenesis of *Drosophila* central nervous system as well as peripheral nervous system. In the development of *Drosophila* SOP lineage, which closely resembles the β and ζ lineages in *C. elegans* male spicule development, down-regulation of the *Notch/Delta* signaling pathway promotes neuronal fate and up-regulation of the pathway results in non-neuronal fate. Yet the *C. elegans Notch* homologue does not seem to play a role in the specification of SPD fate. Neither loss-of-function nor gain-of-function *lin-12* affects SPD marker expression. Activation of *lin-12* pathway at critical developmental time does not influence SPD fate decision. This result does not rule out the possibility that *lin-12* is involved in *C. elegans* male spicule neurogenesis. With the *gpa-1::lacZ* marker only the specification of SPD fate can be assayed. The SPV neuron is derived from β lineage. Although β and ζ lineages share similarities in their cell division pattern, they appear to behave differently. β lineage is

relatively easy to be perturbed or duplicated by removing certain cells in the tail region or under various genetic backgrounds (Chamberlin and Sternberg, 1993, 1994). Thus it is very likely that the mechanism for SPV fate specification is different from that for SPD fate. Study of SPV fate specification will require a molecular marker specific for the SPV fate. With the SPD marker I did find that the Wnt signaling pathway is involved in the specification of SPD fate (Chapter IV). Those mutants that affect SPD fate do not seem to affect SPV fate specification in a similar fashion.

Using molecular marker for genetic screen

Terminal cell fate specification has been difficult to study in *C. elegans* due to the lack of molecular markers and the inability to judge a cell fate based on its nuclear morphology. Therefore, identification of molecular markers for certain cell fates becomes necessary. Expression pattern based genetic screens have been conducted to look for cell migration defective or cell fate specification defective mutants (Chanal and Labouesse, 1997; Forrester et al., 1998; Colavita and Culotti, 1998). To design an expression pattern based genetic screen, several issues need to be taken into consideration. These include the reliability and timing of marker expression, the control for general regulation of gene expression, the association between marker expression and specific phenotypes, alternative markers for mutant analysis, etc..

This study is made possible by the identification of a molecular marker specific for the SPD fate. Since many signaling pathways and mechanisms for cell fate specification are used at multiple time points through development,

using markers to study terminal cell fate specification may reveal late functions of known genes and pathways. The screen may discover new conditional alleles of known genes as well as specific downstream factors of known pathways. The *gpa-1::lacZ* marker is not solely expressed in the spicule region, but also in the phasmid neurons and several cells in the head region. Therefore, it provides an internal control to eliminate general regulators of gene transcription during the genetic screen. Indeed about 50% of potential mutants that were picked after the first round F2 selection turned out to be general regulators of gene transcription and were discarded. *gpa-1::lacZ* expression in the spicules is turned on during L4 molt and stays on in adult males. During the screen all adult males can be included to examine expression pattern, worms need not to be staged carefully. In the spicule region, *gpa-1::lacZ* marker is only expressed in one cell per spicule, making it possible to screen the expression patterns under dissecting microscope. All these features of the marker made this screen reliable and easy to handle.

However, the *gpa-1::lacZ* marker is turned on rather late. The SPD neuron is born at the end of L3 stage. During L4 stage, male tail undergoes complex morphogenetic shaping which involves dramatic changing of cell positions. At this stage it is very difficult to determine cell identities based on cell positions and nuclear morphology. In other words, even with GFP markers it is impossible to tell which cell(s) expresses the GFP marker without doing ablations. Markers that are expressed in early stages prior to the reshaping of male tail will be very helpful to study the cell fate specification as well as morphogenesis during male spicule development.

From the genetic screen I recovered two potentially interesting and specific mutants are *sy549* and *8T9*. *sy549* shows extra marker expressing cell associated with the spicule, while *8T9* displays lack of marker expression in the spicule region. Both of the mutants have wild-type spicule morphology and no other apparent plate phenotypes. Therefore, they are unlikely to be recovered from spicule morphology based screens. *sy549* is further studied in Chapter IV. The specificity of *8T9* remains to be further examined.

Several mutants with various spicule morphology defects were also recovered from the screen. It appears that mutants with early B lineage defect usually have general male tail patterning defect and have severely defective spicules. Whereas mutants with B lineage defect after 10-cell stage would have crumpled spicules to various degrees. There is not much correlation between lineage defect and spicule morphology, or between terminal cell fate and spicule morphology. This makes it difficult to interpret mutant phenotypes based on spicule morphology and marker expression. Markers that are expressed in L3 stage would help solving this issue.

Acknowledgement

I thank Jane Mendel for the PS1701 and PS1702 strains.

REFERENCES:

Avery, L., and Horvitz, H. R. (1987). A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. Cell 51: 1071-1078.

Avery, L., and Horvitz, H. R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous-system of *C. elegans*. Neuron 3: 473-485.

Baumeister, R., Liu, Y. X., and Ruvkun, G. (1996) Lineage-specific regulators couple cell lineage asymmetry to the transcription of the *Caenorhabditis elegans* POU gene *unc-86* during neurogenesis. Gene & Dev. 10: 1395-1410.

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

Chamberlin, H. M., and Sternberg, P. W. (1993). Multiple cell-interactions are required for fate specification during male spicule development in *Caenorhabditis elegans*. Development 118 (2): 297-324.

Chamberlin, H. M., and Sternberg, P. W. (1994). The *lin-3/let-23* pathway mediates inductive signaling during male spicule development in *Caenorhabditis elegans*. Development 120 (10): 2713-2721.

Chamberlin, H. M. (1994). Cell fate specification during *Caenorhabditis elegans* male tail development. Ph. D. thesis. California Institute of Technology.

Chamberlin, H. M., and Sternberg, P. W. (1995). Mutations in the *Caenorhabditis elegans* gene *vab-3* reveal distinct roles in fate specification

and unequal cytokinesis in an asymmetric cell division. Dev. Biol. 170 (2): 679-689.

Chanal, P., and Labouesse, M. (1997). A screen for genetic loci required for hypodermal cell and glial-like cell development during *Caenorhabditis elegans* embryogenesis. Genetics 146: 207-226.

Chisholm, A. D., and Horvitz, H. R. (1995). Patterning of the *Caenorhabditis elegans* head region by the PAX-6 family member *vab-3*. Nature 377: 52-55.

Colavita, A., and Culotti, J. G. (1998). Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in *Caenorhabditis elegans*. Devel. Biol. 194: 72-85.

Finney, M., Ruvkun, G., and Horvitz, H. R. (1988). The *C. elegans* cell lineage and differentiation gene *unc-86* encodes a protein with a homeodomain and extended similarity to transcription factors. Cell 55: 757-769.

Fire, A. (1992). Histochemical techniques for locating *Escherichia-coli* beta-galactosidase activity in transgenic organisms. Genet. Anal. 9: 151-158.

Forrester, W. C., Perens, E., Zallen, J. A., and Garriga, G. (1998). Identification of *Caenorhabditis elegans* genes required for neuronal differentiation and migration. Genetics 148: 151-165.

Hartenstein, V., and Posakony, J. W. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. Dev. Biol. 142 (1): 13-30.

Herman, M.A., and Horvitz, H.R. (1994). The *Caenorhabditis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. Development 120 (5): 1035-1047.

Herman, M. A., Vassilieva, L. L., Horvitz, H. R., Shaw, J. E., and Herman, R. K. (1995). The *C. elegans* gene *lin-44*, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. Cell 83 (1): 101-110.

Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. Nature 377: 627-630.

Liu, K. S., and Sternberg, P. W. (1995). Sensory regulation of male mating behavior in *Caenorhabditis elegans.* Neuron 14 (1): 79-89.

Lochrie, M. A., Mendel, J. E., Sternberg, P. W., and Simon, M. I. (1991). Homologous and unique G-protein alpha subunits in the nematode *Caenorhabditis elegans*. Cell. Regul. 2 (2): 135-154.

Parks, A. L., and Muskavitch, M. A. (1993). *Delta* function is required for bristle organ determination and morphogenesis in *Drosophila.* Dev. Biol. 157 (2): 484-496.

Rhyu, M. S., Jan, L. Y., and Jan, Y. N. (1994). Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76 (3): 477-491.

Roehl, H., and Kimble, J. (1993). Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. Nature 364: 632-635.

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

Sulston, J. E., Albertson, D. G., and Thomson, J. N. (1980). The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. Dev. Biol. 78: 542-576.

Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Devel. Biol.* 78: 577-597.

Yochem, J., Weston, K., and Greenwald, I. (1988). The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila Notch*. Nature 335 (6190): 547-550.

Zhao, C., and Emmons, S. W. (1995). A transcription factor controlling development of peripheral sense organs in *C .elegans*. Nature 373: 74-78.

FIGURES:

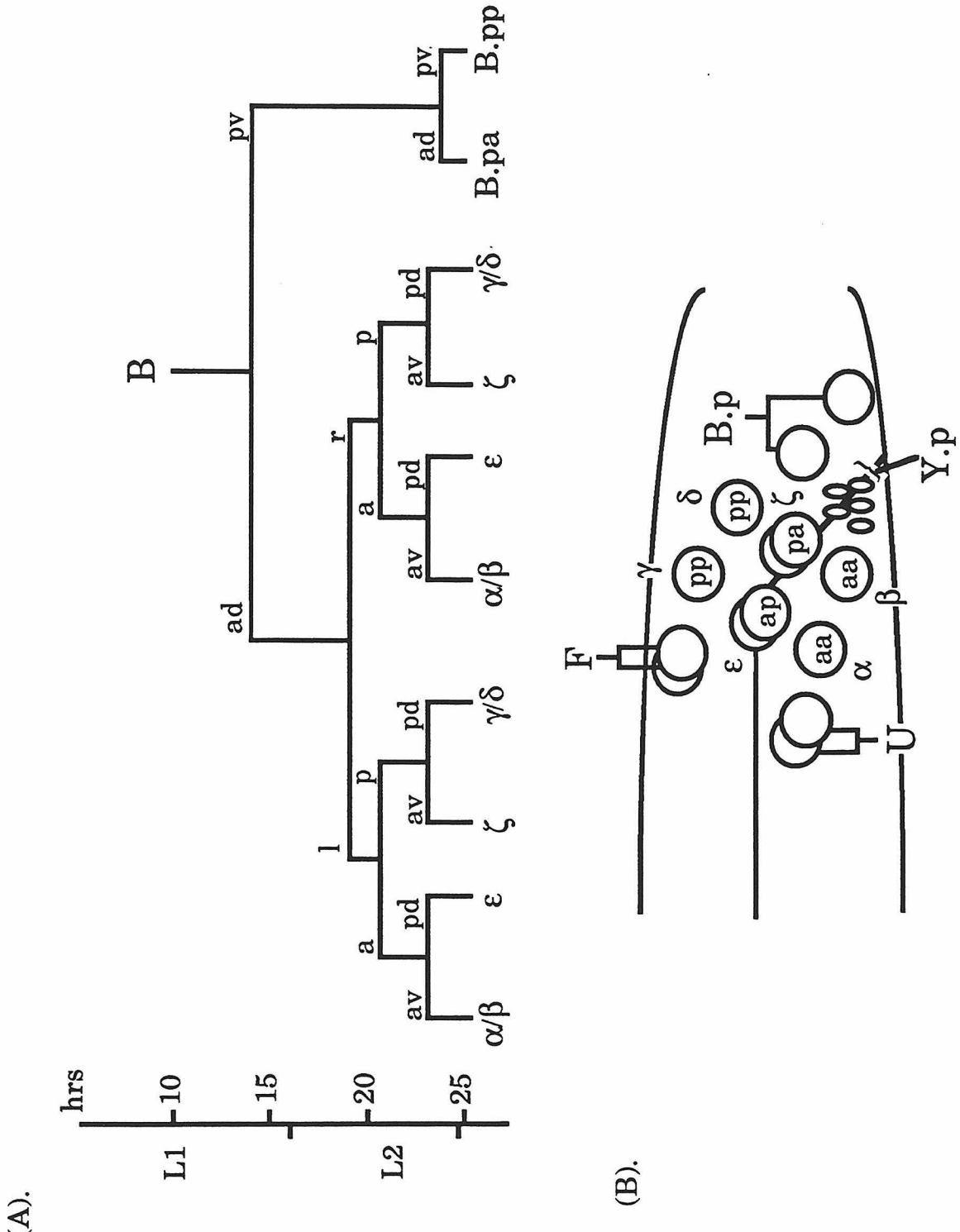
Figure 1. The B lineage.

(A). B lineage of 1-cell to 10-cell stage (taken from Sulston et al. 1980; Chamberlin and Sternberg, 1993).

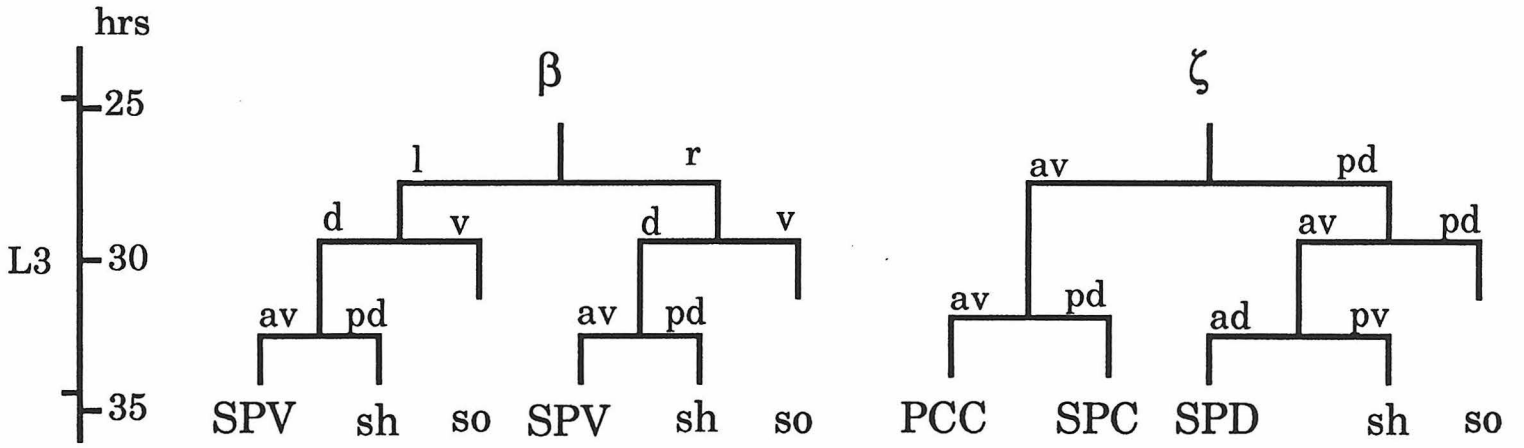
(B). Schematic drawing of cell positions at 10-cell stage.

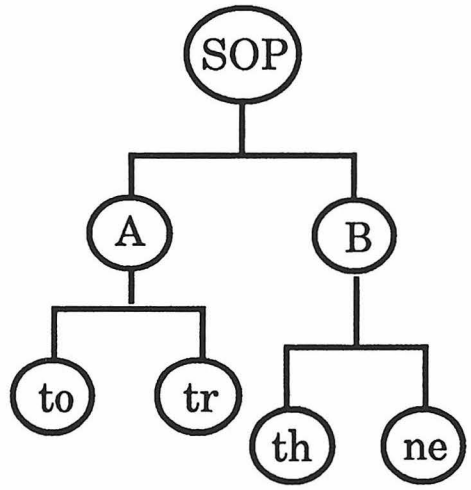
(C). Neurogenic sublineages β and ζ and Drosophila SOP lineage are shown (taken from Sulston et al. 1980; Chamberlin and Sternberg, 1993).

sh: sheath cell; so: socket cell. SPV neuron is derived from β sublineage and SPD neuron from ζ sublineage. SPD neuron and SPD sheath cell are sister cells derived from ζ.pa. Both intercellular molecules Notch/Delta signaling pathway and intracellular factor Numb are involved in the neuronal vs. non-neuronal cell fate decision during the development of SOP lineage.


(A).
hrs
10
15
20
25
L1
L2
B
ad
pv
B.pa
B.pp
l
r
a
p
av
pd
α/β
ε
ζ
γ/δ
(B).
F
U
B.p
Y.p
γ
δ
ε
ζ
α
β
pp
ap
aa
pa

(C).





Notch/Delta
Numb

Figure 2. *gpa-1::lacZ* is expressed in SPD neurons specifically.

(A). wild-type intact animal, 1-cell per spicule expressing the marker.

(B). β and ζ ablated animal, no marker expression in spicules.

(C). β ablated animal.

(D). SPV ablated animal.

(E). ζ ablated animal.

(F). SPD ablated animal.

Strain used for photographs is PS1702. Scale bar indicates 20μm.

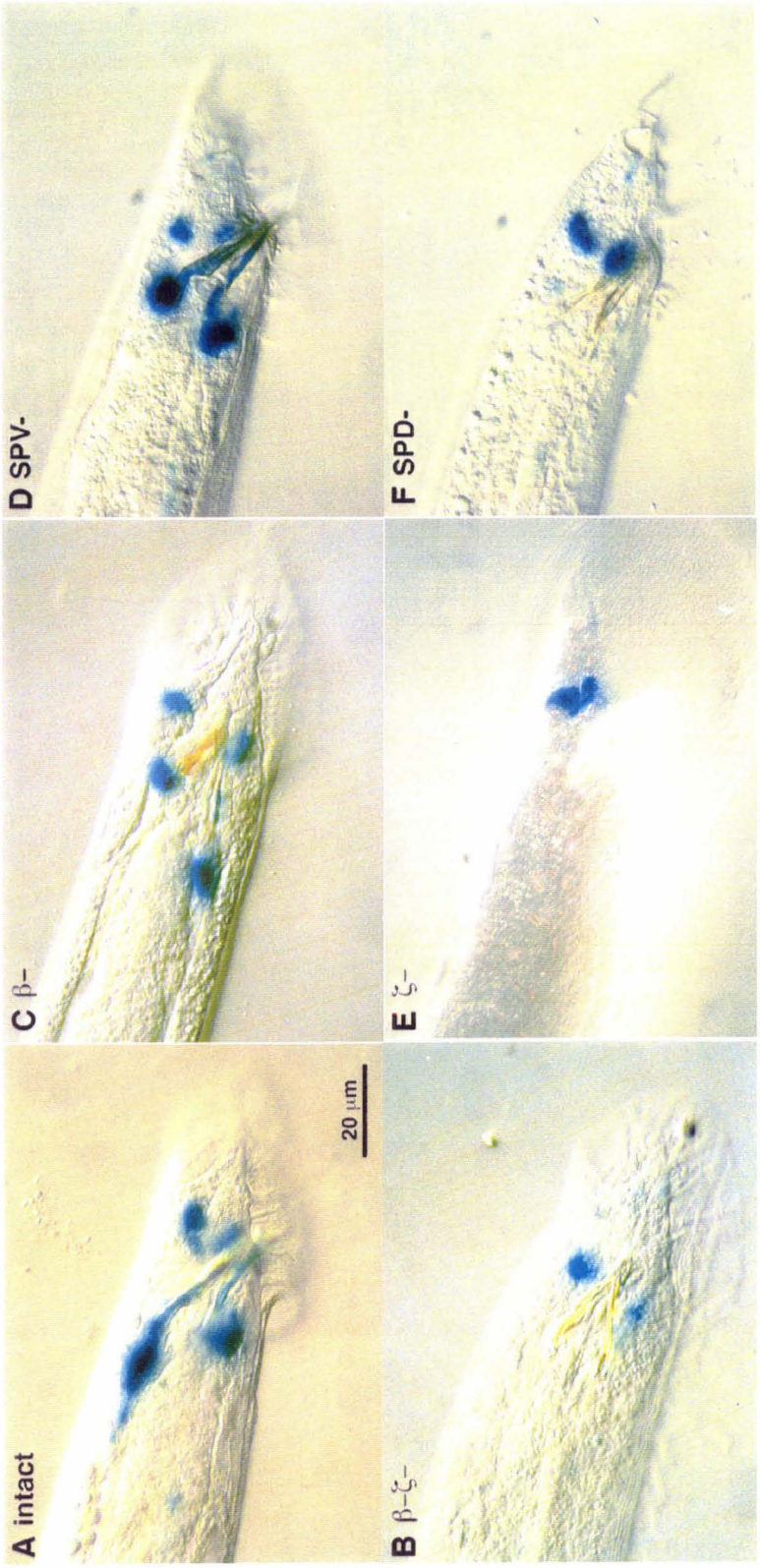

A intact
B β-ξ-
C β-
D SPV-
E ξ-
F SPD-
20 μm

Figure 3. *gpa-1::lacZ* expression in cell(s) ablated animals.

(A). F and U cells were ablated at B lineage 2-cell stage.

(B). F and U cells were ablated at B lineage 10-cell stage.

(C). Y.p cell was ablated at B lineage 2-cell stage.

(D). ζ pair was isolated at B lineage 9-cell to early 10-cell stage by ablating the other 6 B.a progeny.

(E). ζ pair was isolated at late 10-cell stage by ablating the other 6 B.a progeny. Arrowhead indicates two *gpa-1::lacZ* marker expressing cells in the spicule region.

Scale bar indicates 20μm.

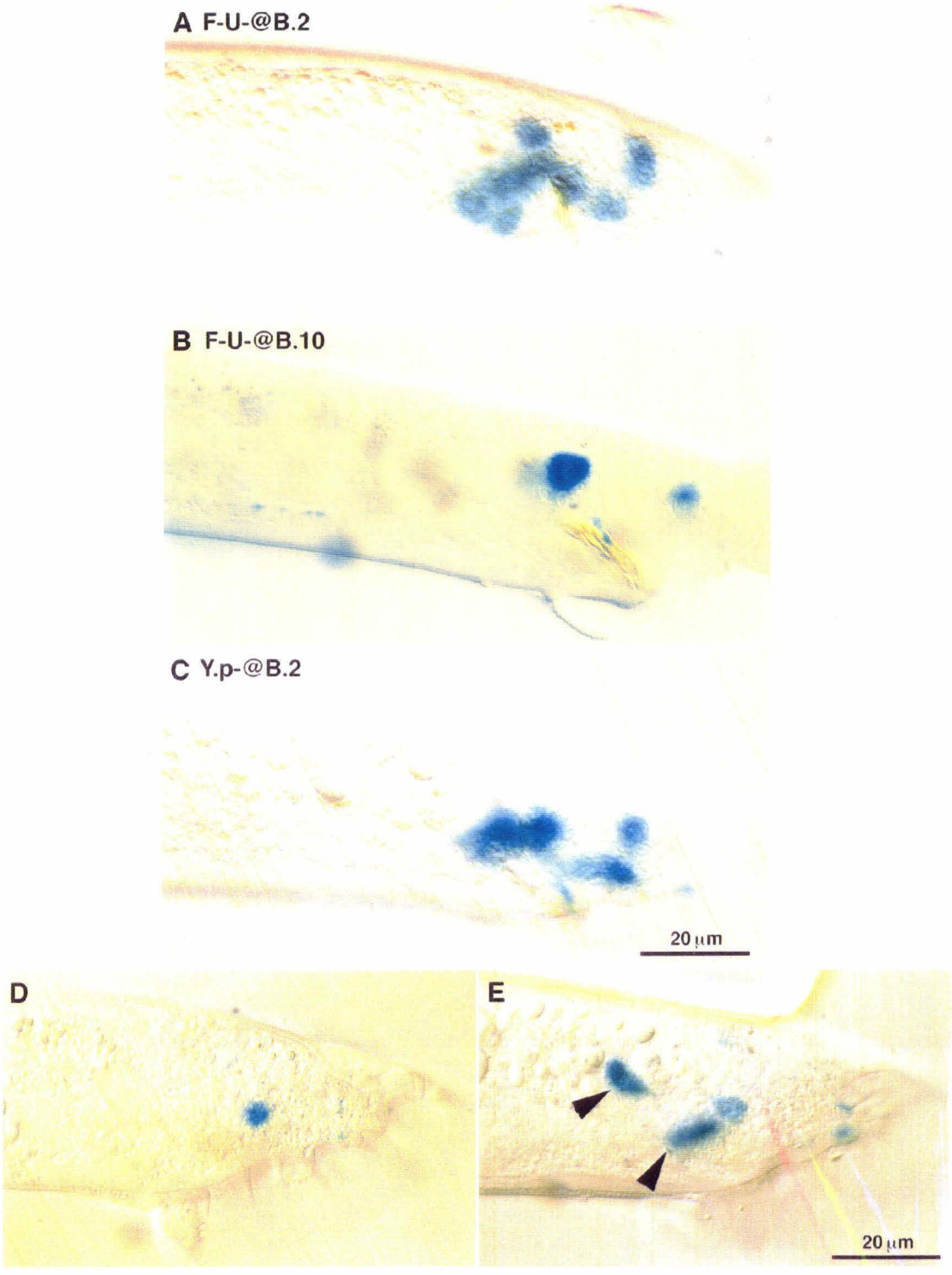

A F-U-@B.2
B F-U-@B.10
C Y.p-@B.2
20 μm
D
E
20 μm

Figure 4. *gpa-1::lacZ* expression in various mutants examined.

(A). *lin-1(e1777)*.

(B). *lin-31(n301)*.

(C). *dpy-19(e1259)lin-12(n676909)*, arrow indicates ectopic hook caused by *lin-12(d)*.

(D). *lin-32(u282)*.

(E). *unc-86(e1416)*.

Scale bar indicates 20μm.

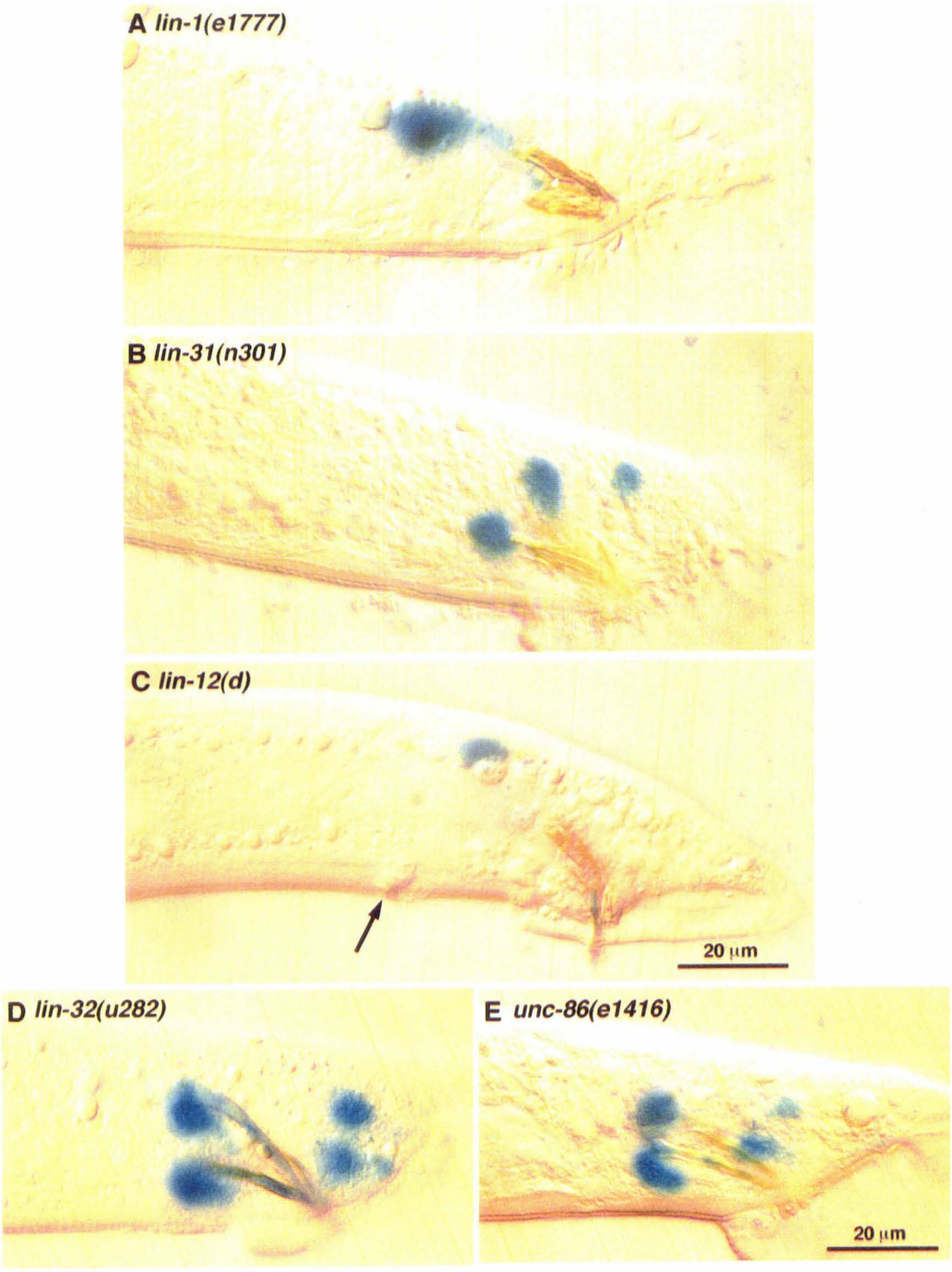

A lin-1(e1777)
B lin-31(n301)
C lin-12(d)
20 μm
D lin-32(u282)
E unc-86(e1416)
20 μm

Figure 5. Screen scheme.

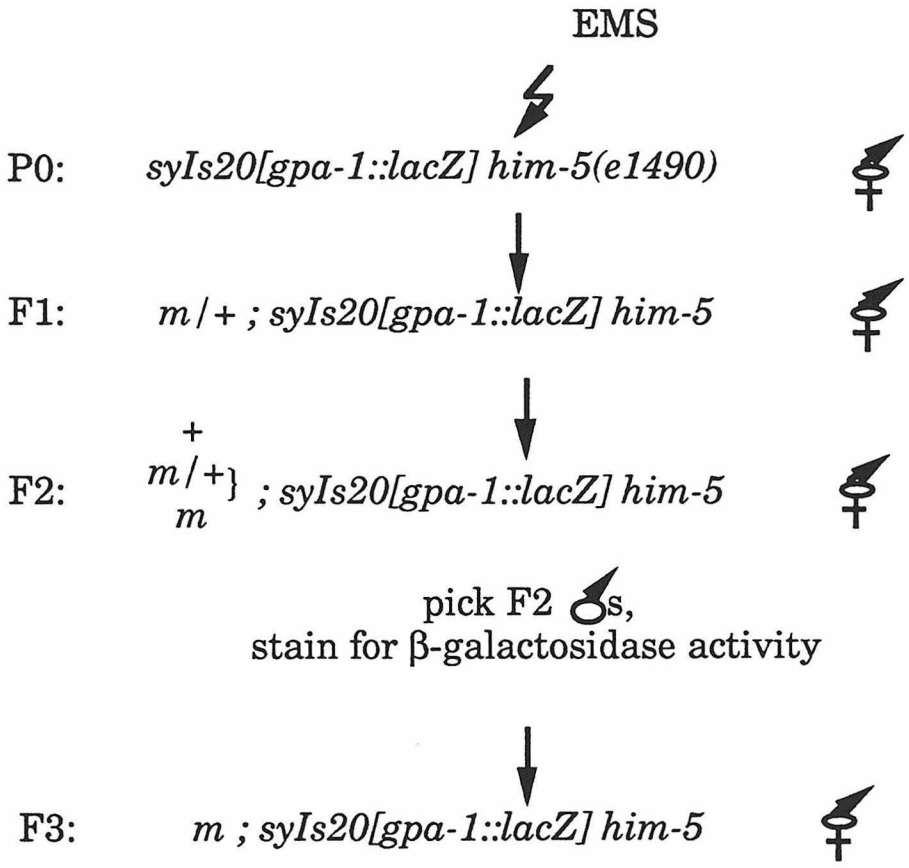


Figure 6. Mutants recovered from the F1 clonal screen.

(A). *2D2*.

(B). *2D2*, excessive marker expression in the spicule region.

(C). *lin-48(sy548)*, loss of marker expression in the spicule region, arrow indicates ectopic spicule material.

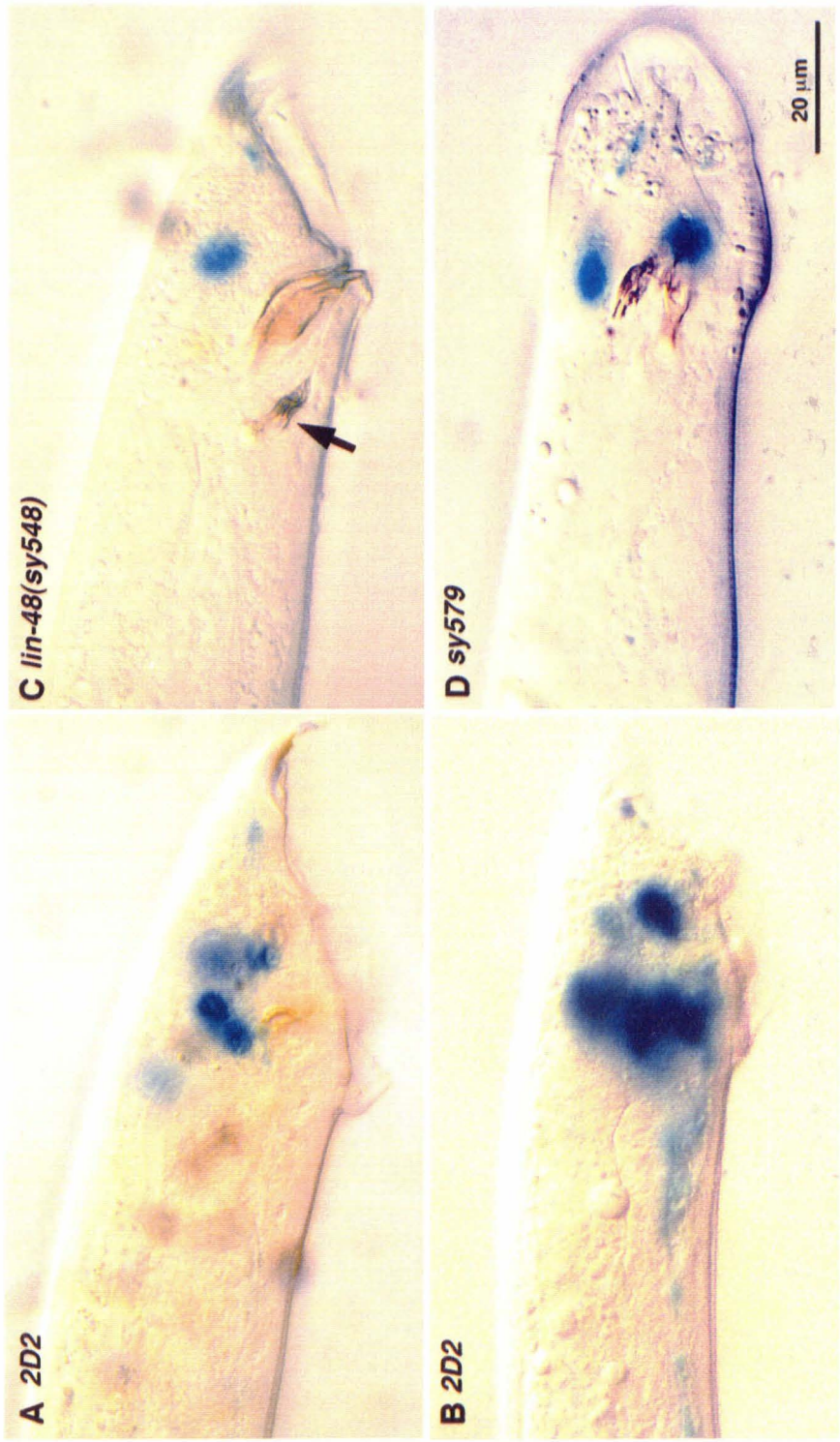
(D). *5U6*, crumpled spicules and loss of marker expression.

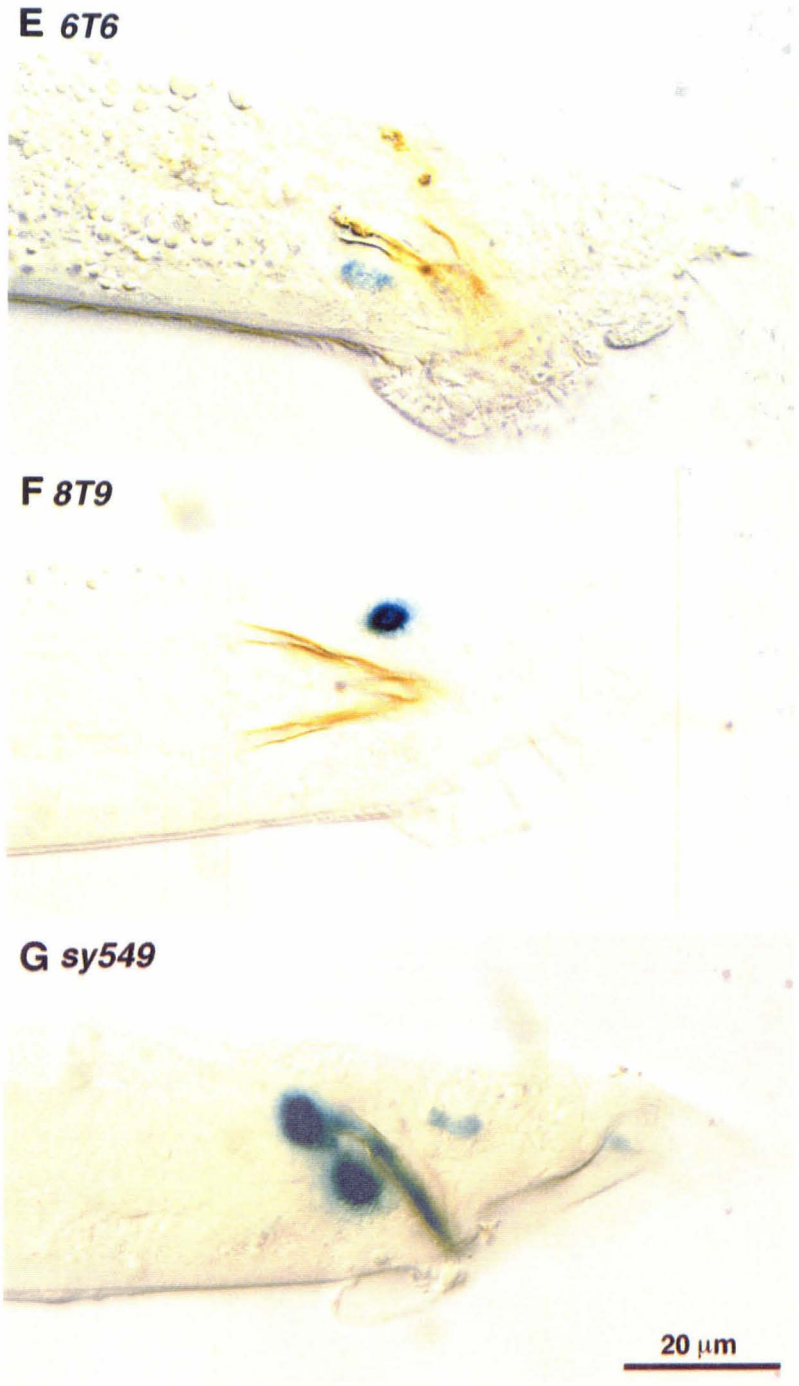
(E). *6T6*, weak marker expression and abnormal spicule cuticle at spicule base region.

(F). *8T9*, loss of marker expression, normal spicule morphology.

(G). *sy549*, 2-cell per spicule expressing *gpa-1::lacZ* marker, lateral view showing left spicule, arrowhead indicates two blue cells.

Scale bar indicates 20μm.


A 2D2
B 2D2
C lin-48(sy548)
D sy579
20 µm


E 6T6
F 8T9
G sy549
20 μm

TABLES:

Table 1. *gpa-1::lacZ* is expressed in SPD neurons specifically.

cells ablated	neurons missing	PS1701	PS1702
		gpa-1::lacZ expression in SPD neurons	*gpa-1::lacZ* expression in SPD neurons
intact	none	all, n>50	all, n>50
β+ζl/r	SPV+SPD, +	0/4	0/3
β	SPV, +	5/5	6/6
βl/r d	SPV, +	5/5	6/6
βl/r da	SPV	8/8	6/6
ζl/r	SPD, +	0/5	2/7*
ζl/r a	SPC, +	4/4	4/4
ζl/r p	SPD, +	1/9*	1/8*
ζl/r pa	SPD, +	1/9*	1/8*
ζl/r paa	SPD	1/10*	1/15*

+: additional cells were removed with the ablation

*: weak staining or only one SPD staining

Table 2. F, U, Y.p ablation.

cells ablated	stage	n	spicule morphology	*gpa-1::lacZ* expression
B	B @ 1-cell st.	2	no spicules	no expression
F + U	B @ 2~3-cell st.	6	5/6 severely crumpled spicules	cluster of extra cells expression
Y.p	B @ 2-cell st.	7	5/7 crumpled spicules	wild-type expression
Fl/r + Ul/r	B @ 10-cell st.	8	7/8 wild-type spicules	wild-type expression

APPENDIX

Other molecular markers that are expressed in the spicule neurons

During the course of this study, we also identified several other markers that are expressed in the spicule neurons and may have potential use for future studies. These markers and their expression patterns are described as below.

gpa-1::GFP

A GFP reporter construct driven by the *gpa-1* promoter displays a different expression pattern than the *gpa-1::lacZ* reporter construct. In the spicule region, it is expressed in all three spicule neurons, SPC, SPD, and SPV. The expression is turned on first in the SPC neuron during the late L4 stage, then in the SPD and SPV neurons during L4 lethargus (Figure 1A, 1B). The *gpa-1::GFP* expression stays on in the spicule neurons in adult males.

sra-1::GFP

sra-1 encodes a seven transmembrane receptor protein (Troemel et al., 1995). Transgenic animals carrying an extrachromosomal array of the *sra-1::GFP* reporter construct was generated. The expression of *sra-1::GFP* is found specifically in the male spicule neurons, most likely SPD and SPV. And the expression is only turned on in adult males.

osm-6::GFP

osm-6::GFP reporter gene has been found to be expressed exclusively in ciliated sensory neurons (Collet et al., 1998). In males, it is expressed in the ray neurons, hook neurons, two spicule neurons, and a neuron associated with the post-cloaca sensilia (p. c. s.) (Collet et al., 1998; M. Barr, personal communication, Figure 1C). The spicule neurons are likely to be the SPD and

SPV neurons. The expression of *osm-6::GFP* in the male tail region is turned on during the late L4 stage to L4 molt, and is turned on in a sequential manner, first in the hook neurons, then in the spicule neurons and p. c. s. neuron, last in the ray neurons. Due to the complexity of the expression pattern, there is only a brief time window during which the spicule neuron expression can be scored clearly.

REFERENCES

Collet, J., Spike, C. A., Lundquist, E. A., Shaw, J. E., and Herman, R. K. (1998). Analysis of *osm-6*, a gene that affects sensory cilium structure and sensory neuron function in *Caenorhabditis elegans*. Genetics 148: 187-200.

Troemel, E. R., Chou, J. H., Dwyer, N. D., Colbert, H. A., and Bargmann, C. I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell 83: 207-218.

FIGURE

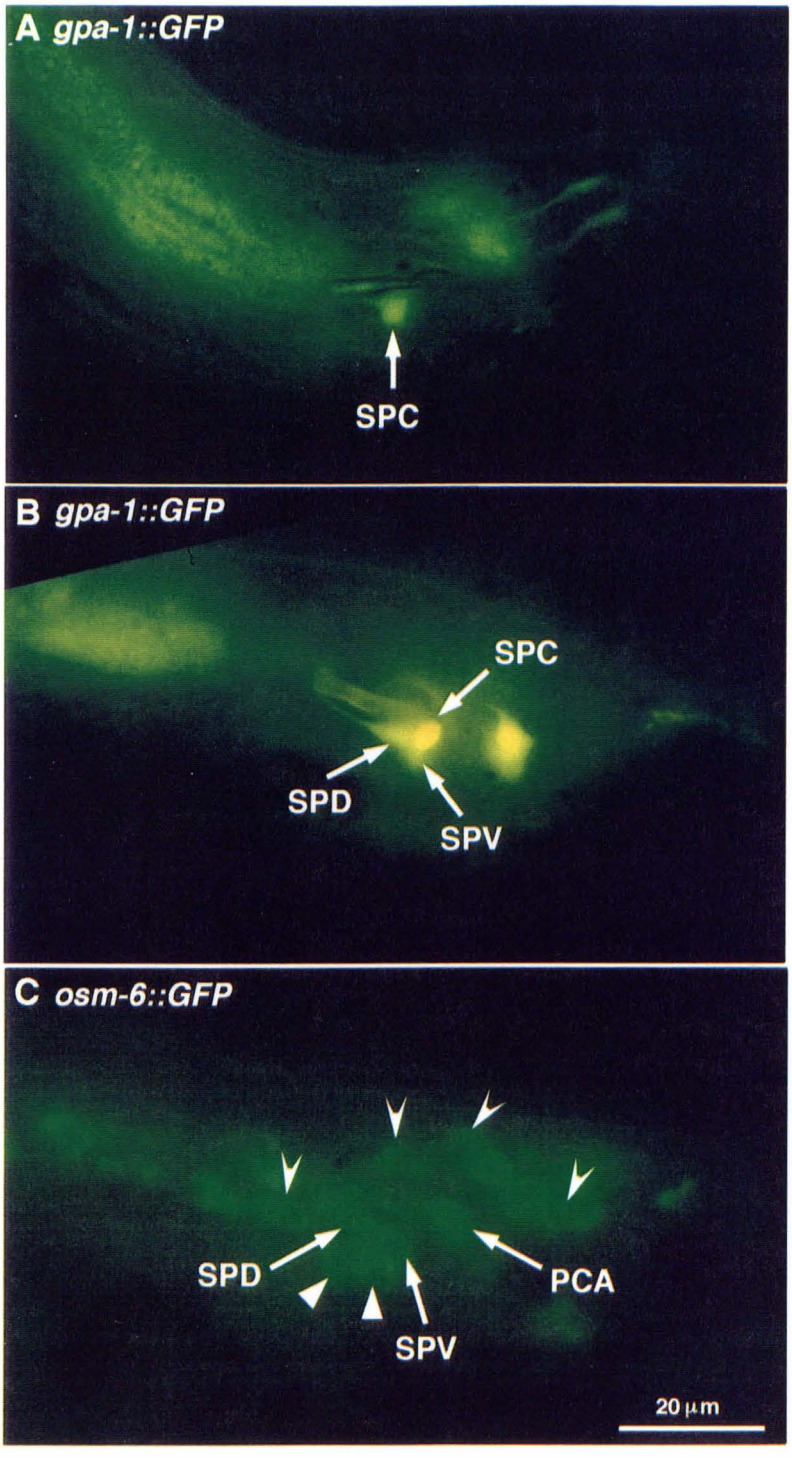
Figure 1. Molecular markers that are expressed in the spicule neurons.

(A). *gpa-1::GFP* expression is turned on in the SPC neuron during the late L4 stage.

(B). *gpa-1::GFP* is expressed in all three spicule neurons, SPC, SPD, and SPV. The cell bodies of these neurons are in a triangle-shaped cluster.

(C). *osm-6::GFP* is expressed in the SPD and SPV neurons. Arrowhead indicates hook neuron, concave arrowhead indicates ray neuron.

Scale bar indicates 20μm.


A gpa-1::GFP
SPC
B gpa-1::GFP
SPC
SPD
SPV
C osm-6::GFP
SPD
PCA
SPV
20 μm

Chapter IV

An HMG1-like protein facilitates Wnt signaling in *C. elegans*

(prepared for submission)

SUMMARY

Using a marker specific for the SPD neuron, we show that during *C. elegans* male spicule development, the specification of glial vs. neuronal cell fate in a canonical neurogenic sublineage is dependent on Wnt signaling. Inactivation of a Wnt signaling pathway mediated by the Wnt receptor LIN-17 transforms the SPD sheath cell into its sister, the SPD neuron. We discovered a new mutant, *son-1*, which displays this same cell fate transformation. The *son-1* mutation enhances the phenotypes of reduction-of-function *lin-17* mutants in several developmental processes, including vulva development, somatic gonad development, and male tail patterning. *son-1* encodes an HMG1/2-like DNA binding protein and is localized in all cell nuclei through development as revealed by a GFP reporter construct. Disruption of *son-1* function by RNA-mediated interference results in the same postembryonic defects as caused by overexpression of POP-1, a TCF/LEF class HMG protein known to act downstream of the Wnt signaling pathway. Our results provide in vivo evidence for the functional involvement of an HMG1/2-like protein, SON-1, in Wnt signaling. The sequence non-specific and sequence specific HMG proteins SON-1 and POP-1 may both act in the Wnt responding cells to regulate gene transcription in opposite directions.

INTRODUCTION

The Wnt signaling pathway is one of the major signaling pathways controlling animal development. It controls cell proliferation, cell and tissue polarity, and cell fate specification in many developmental processes of many organisms. In recent years, several components of the Wnt signaling pathway have been identified and the mechanisms by which these factors act have begun to be elucidated (reviewed in Cadigan and Nusse, 1997; Moon et al., 1997). Wnt family genes, which encode secreted glycoproteins, act as intercellular signals. The Frizzled (Fz) family seven transmembrane proteins are putative Wnt receptors that transduce Wnt signals to downstream targets. Activation of the Wnt signaling pathway leads to accumulation of β-catenin in the cytoplasm. β-catenin interacts with the TCF/LEF family HMG box-containing DNA binding protein and the complex accumulates in the nucleus, whereby regulates gene expression of Wnt pathway targets. Although this simplified model appears to be conserved among diverse organisms, the discoveries of new components and the multiplicity of Wnt pathway components have added complexity to this model.

In *C. elegans*, there are several Wnt signaling pathways that are involved in several aspects of development: cell fate specification during embryogenesis, vulval formation, somatic gonad development, and tail patterning. Some components are shared by several or all of these processes. During embryogenesis, Wnt signaling pathway genes, *mom-2* (Wnt), *mom-5* (Fz), *wrm-1* (β-catenin), and *pop-1* (TCF/LEF), mediate interactions between the P2 and EMS cells (Lin et al., 1995, 1998; Rocheleau et al., 1997; Thorpe et

al., 1997). Signaling from the P2 cell promotes asymmetric division of the EMS cell to produce an anterior MS fate and a posterior E fate. Inactivation of the Wnt signaling pathway results in an E to MS fate transformation. The Wnt pathway also plays a role in vulval development. Mutants of several Wnt pathway components show various vulva lineage defects, for example *lin-17* (Fz), *bar-1* (β-catenin), and *mom-1* and *mom-3* mutants (Sternberg and Horvitz, 1988; Sawa et al., 1996; Eisenmann et al., 1998; Thorpe et al., 1997). In the somatic gonad development, *lin-17* has also been shown to control several asymmetric cell divisions (Sternberg and Horvitz, 1988). In the tail region, a Wnt signal LIN-44 and its putative receptor LIN-17 control the polarity of several asymmetric cell divisions, including B, T, F and U (Herman and Horvitz, 1994; Chamberlin and Sternberg, 1995; Herman et al., 1995; Sawa et al., 1996). *lin-44*, *lin-17* and *bar-1* are also involved in the specification of P12 fate in the tail region (Jiang and Sternberg, 1998; Eisenmann et al., 1998). A common feature of most of these Wnt signaling pathways in *C. elegans* appears that Wnt signaling is required for posterior cell fates and inactivation of Wnt signaling results in posterior to anterior fates transformation.

The TCF/LEF class HMG box proteins have been shown to regulate Wnt target gene expression upon pathway activation (van de Wetering et al., 1997). The *C. elegans* TCF/LEF class protein is encoded by the *pop-1* gene (Lin et al., 1995). *pop-1* negatively regulates Wnt signaling, as loss-of-function mutation in *pop-1* confers an opposite phenotype as inactivation of Wnt signaling. Moreover, the expression level of POP-1 protein appears to be up-regulated upon inactivation of Wnt signaling (Rocheleau et al., 1997; Lin

et al., 1998). Therefore, it was proposed that activation of the Wnt signaling pathway relieves the inhibitory effects of POP-1 on gene transcriptional regulation. Yet it is not clear how Wnt target gene expression is regulated and what other unidentified factors are involved in this process.

We have been studying cell-cell interactions and cell fate specification during *C. elegans* male spicule development. Previous studies have shown that cell fate specification in spicule development is mediated by multiple signaling pathways. The *C. elegans* male spicule is generated by a single male specific B blast cell (Figure 1). The first division of B cell is asymmetric and is controlled by the Wnt signaling pathway genes, *lin-44* and *lin-17* (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Chamberlin and Sternberg, 1995). At the early third larval stage (L3; B lineage 10-cell stage), the specification of B cell progeny involves a RAS signaling pathway and a *C. elegans* Notch homologue *lin-12* (Chamberlin and Sternberg, 1994). During the L3 stage, those cells continue to divide to generate spicule neurons, non-neuronal sheath and socket cells, and connective tissues (Figure 1). For example, the ζ sublineage generates the neuron SPD and its associated sheath (SPDsh) and socket (SPDso) cells.

Using a molecular marker that is specifically expressed in the ζ-derived SPD neurons, we studied neuronal vs. non-neuronal cell fate specification during male spicule development. Although the cell division pattern of the ζ sublineage resembles the *Drosophila* sensory organ precursor (SOP) lineage in which the Notch pathway plays a crucial role, we found that *lin-12* is not involved in the specification of the SPD neuron fate. Rather the

lin-17-mediated Wnt signaling pathway plays an important role in this neuronal vs. non-neuronal fate decision in the ζ lineage. Loss-of-function mutants of *lin-17* display a SPDsh to neuron fate transformation. To identify new genes involved in this cell fate decision, we conducted a genetic screen and discovered a mutant, *son-1(sy549)*, which shows the same cell fate transformation. Moreover *son-1* mutation enhances the phenotypes of a *lin-17* hypomorph, *n698*, to *lin-17* null-like, with respect to vulval development, somatic gonad development, and male tail patterning. We cloned the *son-1* locus and found it encodes an HMG1/2-like DNA binding protein, which is distinct from the TCF/LEF class HMG proteins. Disruption of *son-1* function through RNA-mediated interference (*RNAi*) leads to defects in several Wnt pathway mediated developmental processes. Overexpression of POP-1 causes the same spectrum of phenotypes as *son-1 RNAi*. Our results provide in vivo evidence that the HMG1/2 like protein encoded by *son-1* plays a specific role in Wnt signaling. SON-1 and POP-1 may both act in the Wnt responding cells but in opposite directions to regulate gene transcription. Taken together, these data provide evidence for further complexity in Wnt responses during development.

RESULTS

lin-17 mutants cause a SPDsh to neuron fate transformation

To study terminal cell fate specification in *C. elegans* male spicule development, we used a molecular marker, *gpa-1::lacZ*. In transgenic animals bearing a *lacZ* reporter gene driven by the *gpa-1* promoter, expression is

found in male spicules as well as phasmid neurons in the tail region and several cells in the head (J. Mendel and P. W. Sternberg, unpublished). In wild-type animals *gpa-1::lacZ* marker is expressed in one cell per spicule. Cell ablation experiments identified this cell as the spicule neuron SPD (Figure 2A, Chapter III).

Using this SPD neuron specific marker, we examined spicule terminal cell fates in several classes of known mutants. Although the cell division pattern of the spicule neurogenic lineage resembles that of the *Drosophila* SOP lineage, we found that the *C. elegans* Notch homologue, *lin-12*, is not involved in the specification of the SPD neuron vs. SPDsh fate (Chapter III).

The Wnt pathway genes, *lin-44* and *lin-17*, are involved in the first asymmetric division of the B cell. Mutant males bearing strong *lin-44* and *lin-17* alleles have early B lineage defects, resulting in severely crumpled spicules. From a few escapers where the first division of B cell is not disrupted, we noticed extra expression of the *gpa-1::lacZ* marker in the spicule region (Figure 2B, 2C). Using a weak *lin-17* allele, *n698*, wherein the first B cell division is usually not disrupted, we found that 80% of the animals show two cells per spicule expressing the SPD marker (Figure 2D, Table 1).

To determine which cell is responsible for the ectopic expression, we ablated specific cells in the B lineage and examined marker expression in operated animals. Since the ε lineage develops abnormally in *lin-17* mutants (Chamberlin and Sternberg, 1995; this study), we first ablated the ε cells at 10-cell stage and examined the marker expression pattern in operated

animals. Ablation of ε does not eliminate the extra *gpa-1::lacZ* marker expressing cell (n=9). Further ablation experiments indicate that the extra expression results from SPDsh, the sister cell of SPD neuron (Table 1). Ablation of the precursor cell of SPD and SPDsh eliminated marker expression in the spicule region. Ablation of either cell resulted in one cell per spicule expressing the marker. We also used other cell type specific markers to examine the terminal cell fates in *lin-17(n698)* mutants. *osm-6::GFP* is normally expressed in SPD and SPV neurons of wild-type spicules and in male sensory ray neurons (Collet et al., 1998). In the *lin-17(n698)* mutant, *osm-6::GFP* is expressed in an extra cell associated with the spicules (4/6 animals examined), consistent with the extra expression observed using the *gpa-1::lacZ* marker. Expression of a socket cell marker, *egl-17::GFP* (Burdine et al., 1998; Chapter V), was not affected by the *lin-17(n698)* mutation (n=10), suggesting that the socket cells are correctly specified in *lin-17(n698)* mutants. Therefore, *lin-17* mutants confer a sheath to neuron fate transformation.

son-1 mutant confers the same sheath to neuron fate transformation

To discover additional loci involved in the neurogenic sublineage cell fate specification, we performed an F1 clonal screen looking for mutations that alter *gpa-1::lacZ* expression specifically in the spicule region. From the screen, we recovered one mutant, *sy549*, in which two cells per spicule express the *gpa-1::lacZ* marker (Figure 2E). Cell ablation experiments revealed that the extra cell that expresses the *gpa-1::lacZ* marker is the SPDsh cell, as in *lin-17* mutants (Figure 2E-2G, Table 1).

sy549 mutant males have wild-type spicules and wild-type B cell division pattern. Expression patterns of other cell type specific markers examined are consistent with the conclusion that *sy549* confers a SPDsh to SPD neuron fate transformation (*osm-6::GFP*: 7/11 animals showed extra marker expression in the spicule region; *egl-17::GFP*: 10/10 wild-type expression). We thus name this mutant *son-1* for sheath-to-neuron fate transformation.

son-1(sy549) displays several phenotypes resembling lin-17 mutants

In addition to the male spicule phenotype, *son-1(sy549)* mutants also display a protruding vulva phenotype with high penetrance. Hermaphrodites retain eggs in uterus, some animals have a disorganized gonad and explode at the vulva, and the brood size is low (~50 in a *syIs20 him5(e1490)* background). Abnormal vulval lineages and vulval morphology are observed in about 10% of animals (n>50). The abnormal vulval lineage occurs in 2° cells; it can occur at either P5.p or P7.p. In most cases, the correct number of cells were generated but the vulval morphology was abnormal, and an extra vulva invagination was observed in several animals. This phenotype is similar to the vulval defect observed in *lin-17* mutants (Ferguson and Horvitz, 1985; Ferguson et al., 1987). Occasionally *son-1(sy549)* hermaphrodites have gonads with a single arm instead of two, as observed in *lin-17* mutants (Sternberg and Horvitz, 1988). In addition, like *lin-17* mutant males, *son-1(sy549)* mutant males have ray defects and cannot mate.

son-1(sy549) enhances lin-17(n698) phenotypes to lin-17 null-like

To examine the interactions between *son-1* and *lin-17*, we constructed strains defective in both *son-1* and *lin-17*. We first examined the *gpa-1::lacZ* expression pattern in the double mutant animals. The SPD neuron marker is expressed in no more than two cells per spicule in the double mutants, confirming the conclusion from the ablation experiment that both of them confer the same sheath to neuron fate transformation.

lin-17 mutants are defective in vulva formation, somatic gonad development, and phasmid and spicule development in the tail. *n698* is a reduction-of-function allele of *lin-17* (Sawa et al., 1996) and displays less strong phenotypes than a null allele, *n671*. About 50% of *n698* animals have abnormal vulval invaginations, but the extra posterior invagination phenotype is rarely seen (Table 2). By contrast, all *n698; sy549* double mutant animals examined have abnormal vulval invaginations, and ~70% of animals show an extra posterior invagination, which is typically seen only in strong *lin-17* alleles (Table 2). ~73% *n698* animals have a T lineage defect such that their phasmids fail to take up DiO (Sawa et al., 1996). *son-1(sy549)* mutants are wild-type for DiO staining, while 98% of *n698*; *sy549* double mutant animals fail to uptake DiO (Table 2). Double mutant hermaphrodites often have a single gonad arm instead of the normal two. Double mutant males show ectopic meiosis, indicative of no distal tip cells (Sternberg and Horvitz, 1988). Double mutant males also show more severely crumpled spicules and have no rays. Taken together, *lin-17(n698); son-1(sy549)* double mutant animals behave like strong *lin-17* mutant animals with respect to vulva development, somatic gonad development, and male tail patterning.

However, neither *lin-17(n698)* mutants nor the double mutants exhibit a P12 fate specification defect (Table 2).

We further constructed a double mutant strain carrying *son-1(sy549)* and a *lin-17* null allele, *n671*. We examined two relatively low penetrance phenotypes, the ectopic posterior vulva invagination phenotype and P12 fate specification. We did not observe any phenotypic enhancement in the double mutant animals (Table 2). Therefore, *son-1* and *lin-17* likely act in the same pathway.

son-1 encodes an HMG1/2-like protein

son-1(sy549) is a recessive mutant. Based on the spicule phenotype, we mapped it to chromosome III, between *lon-1* and *unc-36* (Figure 3). Deficiency mapping put *son-1(sy549)* between the left end point of *sDf127* and the left end point of *nDf16*. Hemizygous males (*sy549/Df*) show a similar defect in *gpa-1::lacZ* marker expression as *sy549* homozygotes. Hemizygous hermaphrodites appear to be sterile possibly as a result of disorganized somatic gonad and bursting at the vulva. Therefore, *sy549* is likely to be a reduction-of-function mutant.

We obtained germline transformation rescue of *son-1(sy549)* by a single cosmid C18F10 that maps in this region of chromosome III (Figure 3). A 3kb PCR fragment amplified from this cosmid was able to fully rescue all the mutant phenotypes (Figure 3). A single gene F47D12.4 is predicted within this 3kb genomic region. We performed RT-PCR with primers specific to the predicted 5' start and 3' stop codon regions of this gene and obtained cDNA clones of about 600bp. Sequences of five clones revealed that three

clones are the same as predicted by Genefinder program; the other two clones appear to be generated through alternative splicing at the end of the first exon, which adds two additional amino acids without changing the rest of the protein coding. The 5'-terminus of the gene was further confirmed by RT-PCR using an SL1 primer and an internal gene-specific primer.

This gene encodes an HMG1-like protein (Figure 4A). It contains two HMG boxes, box A and box B, and is most homologous to the HMG1/2-like proteins of *Drosophila*, mouse and human, exhibiting ~55% identity within the HMG boxes. It also displays ~25% identity to TCF/POP/SOX family proteins within the HMG box (Figure 4B).

We sequenced the *sy549* allele and identified a missense mutation of g→a at nucleotide position 228, which changes the amino acid 72 from aspartic acid (D) to asparagine (N) (Figure 4A). This amino acid change occurs at a conserved residue in the middle of the third helix of HMG box A.

son-1::GFP is expressed in all somatic cell nuclei through development

We examined the expression pattern of *son-1* by generating both transcriptional and translational fusions of *son-1* to a GFP reporter gene. The constructs contain either 1kb or 3-4kb upstream genomic sequence of *son-1*. We found that *son-1* is expressed in every somatic cell through development (Figure 5). The transcriptional fusion construct contains only part of HMG box A and does not show nuclear localization of GFP. The translational fusion construct is predicted to encode a fusion protein of full length SON-1, lacking the last 6 amino acids, followed by GFP. This construct is able to rescue *son-*

1(sy549) phenotypes and displays nuclear localized GFP expression. This expression pattern is consistent with the predicted role for HMG-box containing proteins as DNA binding proteins.

son-1(sy549) mutants are not generally defective in gene transcription

The HMG1/2-like proteins have been traditionally considered as architectural components of chromatin that have general roles in regulating chromosomal functions. Although several recent studies have started to suggest specific roles for the HMG1/2-like proteins in regulating gene transcription (Zwilling et al., 1995; Zappavigna et al., 1996; Boonyaratanakornkit et al., 1998; Jayaraman et al., 1998), the in vivo functions of this class HMG proteins during development have not been studied. In spite of the broad expression pattern revealed by the *son-1::GFP* reporter gene, we do not think that *son-1* is a general regulator of gene transcription. First, *son-1(sy549)* mutants display specific phenotypes in several developmental processes that resemble *lin-17* mutants, and *son-1(sy549)* genetically interacts with *lin-17*.

Second, *son-1(sy549)* mutant males display ectopic transgene expression specifically in the spicule region. If it were a general regulator of transcription, this phenotype would suggest that loss-of-function *son-1* might also upregulate expression of the *gpa-1::lacZ* reporter elsewhere. Yet we did not observe ectopic or stronger expression of the *gpa-1::lacZ* marker in the phasmid neurons or in the head region. Furthermore, other transgenic lines examined did not show any up-regulation or down-regulation of reporter gene expression by *son-1(sy549)*.

Third, *son-1(sy549)* enhances the phenotypes of a weak allele of *lin-17* to null-like phenotypes. This suggests that loss-of-function *son-1* may down-regulate gene expression. To further test this possibility, we used a sensitized genetic assay that is able to detect a two-fold difference in gene expression (Meneely and Wood, 1987). We used a dosage sensitive allele of the *let-2* gene that is involved in developmental processes unrelated to those mediated by *son-1*. The *let-2* gene encodes a type IV α2 collagen, and its different alleles cause animal lethality with varying severity (Sibley et al., 1994). The allele *mn114* causes a strong dosage-dependent phenotype: animals homozygous for *mn114* can survive to adulthood but are sterile, while hemizygous animals die at larval stages (Meneely and Wood, 1987). If *son-1* is a general regulator of transcription, it should enhance the phenotype of *let-2(mn114)*. Animals homozygous for both *let-2(mn114)* and *son-1(sy549)* can still survive to adulthood as do *let-2(mn114)* homozygotes. Taken together, our results provide the first evidence that the HMG1/2-like proteins have specific developmental functions in vivo.

Elimination of son-1 function by RNAi results in defects in several lin-17-mediated developmental processes

To obtain possible null phenotypes of *son-1*, we disrupted *son-1* function by RNA-mediated interference. dsRNA were synthesized using a full length cDNA clone as template and were injected into adult hermaphrodites. We found an embryonic lethal phenotype as well as postembryonic abnormalities. The embryonic lethality is about 11% penetrant. The reason for the embryonic lethality is not apparent to us. The dead eggs did not show

lack of endoderm fates as seen in *mom* mutants (Rocheleau et al., 1997). Viable hermaphrodites and males grow to adulthood and show a sterile phenotype at ~30% penetrance. Hermaphrodites often have a single gonad arm, resembling the *lin-17* phenotype (Figure 6A; Sternberg and Horvitz, 1988). In males, ectopic meiosis is seen in germline, indicative of loss of distal tip cells (Figure 6B; Sternberg and Horvitz, 1988). This defect is also seen in *lin-17* mutant males a result of Z1 and Z4 lineage defect in which more linker cells are generated at the expense of distal tip cells (Sternberg and Horvitz, 1988). Disruption of *son-1* function by *RNAi* also resulted in a vulval lineage defect and bursting at the vulva region at low penetrance. Abnormal *gpa-1::lacZ* marker expression at the spicule region mimicking *sy549* phenotype was also observed (Figure 6C). Therefore, the phenotypes produced by *son-1 RNAi* are consistent with those seen in *son-1(sy549)* mutants. The *sy549* allele likely reduces but does not fully eliminate SON-1 functions, consistent with the deficiency result. The strong resemblance of *son-1* and *lin-17* mutant phenotypes further suggests that *son-1* is specifically involved in the *lin-17*-mediated Wnt signaling.

Overexpression of POP-1 results in the same spectrum of phenotypes as son-1 RNAi

The TCF/LEF class of HMG box containing proteins, different from the HMG1/2 class of proteins, has been implicated as a component of Wnt signaling pathway to regulate Wnt target gene expression (van de Wetering et al., 1997). The *pop-1* gene encodes a *C. elegans* homologue of the TCF/LEF class HMG protein and has been implicated in Wnt signaling during

embryogenesis (Lin et al., 1995, 1998; Rocheleau et al., 1997; Thorpe et al., 1997). As *pop-1* is thought to negatively regulate Wnt signaling, we wondered if overexpression of POP-1 would result in similar defects as loss-of-function of *son-1*. Indeed, when POP-1 is overexpressed in wild-type animals, we observed similar phenotypes as those seen in *son-1 RNAi* experiments. When POP-1 is overexpressed at L1-L2 stage, somatic gonad defect was observed in both hermaphrodites and males (Figure 6D, 6E). Hermaphrodites have gonad with a single arm, and males show ectopic meiosis in the gonad. When POP-1 is overexpressed at early to mid L3 stage, males show two cells per spicule expressing the SPD marker, resembling the *son-1(sy549)* mutant phenotype (Figure 6F). Thus, the two HMG box containing proteins, POP-1 and SON-1, appear to have opposite roles in regulating Wnt signaling. POP-1 negatively regulates Wnt signaling, while SON-1 positively regulates Wnt signaling.

pop-1 mutant has a maternal effect embryonic lethal phenotype. Animals produced by heterozygous mother that completely lack zygotic *pop-1* are viable and develop normally. Using the spicule SPD neuron specific marker, we found that *pop-1* mutant males have a wild-type marker expression pattern in the spicules. We further examined the genetic interactions between *pop-1* and *son-1*. Mutant animals defective in both genes display additive phenotypes. They still exhibit maternal effect embryonic lethality, while all the postembryonic phenotypes resemble *son-1(sy549)* mutants. Therefore, the *pop-1* gene may not be involved in the postembryonic developmental processes regulated by the *lin-17*-mediated Wnt signaling per se. Rather, those *lin-17*-dependent postembryonic

developmental processes require *son-1* activity with possible involvement of a POP-1-like activity.

son-1 does not act upstream of Wnt

To determine how *son-1* is involved in Wnt signaling, we first tested if *son-1* acts in the Wnt signaling cells or in the responding cells. The Wnt signal LIN-44 is expressed in the tail region and plays a role in tail patterning (Herman and Horvitz, 1994; Herman et al., 1995). We think that LIN-44 is the signal that acts through the LIN-17 receptor during male spicule development. First, during the first division of B lineage, LIN-44 acts through LIN-17 to mediate the asymmetric cell division (Chamberlin and Sternberg, 1995). Second, *lin-44* mutants exhibit a similar SPD neuron marker expression pattern in the spicule region as *lin-17* and *son-1* mutants. We reasoned that if *son-1* acts in the signaling cells to regulate *lin-44* expression, we should be able to rescue *son-1* mutant phenotypes by overexpression of the Wnt signal LIN-44. To test this, we used a construct containing full length *lin-44* cDNA driven by a heat shock promoter. This construct has been shown to rescue *lin-44* phenotypes (Herman et al., 1995). When LIN-44 overexpression is induced during the L2/L3 stage in *son-1(sy549)* mutant background, no suppression of the spicule phenotype is observed. This result indicates that *son-1* does not act upstream of the Wnt signal. Therefore *son-1* is likely to act in the responding cells to regulate Wnt pathway gene expression.

We then tested if overexpression of SON-1 is able to suppress *lin-17* mutant phenotypes. We engineered a *hs-son-1* construct that contains a

genomic clone encoding full length *son-1* driven by a heat shock promoter. In a *son-1(sy549)* background, overexpression of SON-1 by a single pulse of heat shock at the L3 stage within four hours prior to the cell division is able to rescue the spicule sheath to neuron fate transformation phenotype of the mutant. In a *lin-17(n698)* mutant background, we induced SON-1 expression during embryogenesis to the L1/L2 stage and at the L2/L3 stage and examined the phasmid and spicule phenotypes respectively. We did not observe any suppression of *lin-17* phenotypes by SON-1 overexpression. Two possibilities exist. *son-1* might act at the level of Wnt receptor to regulate *lin-17* function. Alternatively, *son-1* activity may not be rate limiting for Wnt pathway function. Consistent with this, simply overexpressing SON-1 in a wild-type background by heat shock or injection of the wild-type gene at high dosage does not result in any gain-of-function phenotype.

Structure/function analysis of SON-1 protein

Both HMG boxes A and B of the HMG1/2-like proteins have been shown to be able to bind bent DNA structures and to interact with certain transcription factors independently in vitro (Bianchi et al., 1992; Zappavigna et al., 1996). To examine the in vivo functions of SON-1 HMG boxes A and B, we engineered deletion constructs lacking either individual box and generated transgenic animals bearing these constructs (Figure 7). In a wild-type background we did not observe any dominant negative or gain-of-function effect caused by these constructs. When these transgenes are crossed into a *son-1(sy549)* mutant background, only the construct containing both HMG boxes is able to rescue the mutant phenotypes. None of the constructs

containing a single HMG box were able to rescue the mutant phenotypes (Figure 7). This result suggests that both HMG boxes A and B are required for the function of the SON-1 protein in vivo.

DISCUSSION

Wnt signaling in spicule development

During *C. elegans* male spicule development, the Wnt pathway genes *lin-44* and *lin-17* control the first cell division of the spicule lineage, namely the B lineage (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Chamberlin and Sternberg, 1995; Sawa et al., 1996). The first division of the B cell, B.a vs. B.p, is asymmetric. Inactivation of the Wnt pathway results in reversed polarity or posterior B.p to anterior B.a fate transformation. Additional functions for *lin-17* have also been implicated in several asymmetric cell divisions in both B.a and B.p lineages after the 10-cell stage (Chamberlin and Sternberg, 1995). Using a terminal cell fate marker, we discovered that the Wnt pathway genes *lin-17* and likely *lin-44* are involved in the fate specification of SPD neuron (ζ.xxa) vs. SPDsh (ζ.xxp) during the last round cell division of the B lineage. Loss-of-function mutation in *lin-17* results in a posterior sheath to anterior neuron fate transformation. Therefore, the *lin-17*-mediated Wnt signaling pathway is used at the first and the last cell division of B cell development to mediate cell fate specification. Like most of the other Wnt-mediated developmental processes, the Wnt pathway acts to promote the posterior fate in the *C. elegans* male spicule lineage. We envision that the source of Wnt signal LIN-44, the hyp8-11 cells

at the tip of the tail (Herman et al., 1995), sets up the anterior and posterior polarity for general tail patterning.

In *C. elegans*, several Wnt pathways mediate several distinct developmental processes. Some components are unique, others are shared in several processes. We examined the possible involvement of other Wnt pathway components in the last cell division of male spicule development. In *lin-44* mutant males, the abnormal SPD neuron marker expression in the spicule region is observed, resembling that of the *lin-17* mutants. However, all the *lin-44* alleles available are null or strong alleles, wherein the early B lineage defect is highly penetrant, making it difficult to rigorously examine its effect on the later stage cell fate specification. *mom-1* and *mom-3* homozygous males display a severely crumpled spicules phenotype at high penetrance, indicating an early B lineage defect. For two *bar-1* alleles tested, *sy324* and *mu349*, the *gpa-1::lacZ* marker expression appeared to be normal in mutant males. *pop-1* mutant males have wild-type spicules and normal marker expression pattern. However, overexpression of POP-1 at the L3 stage results in extra cell expressing *gpa-1::lacZ* in the spicules, similar to loss-of-function *son-1* mutants.

During the development of *C. elegans* male spicules, the neurogenic sublineages β and ζ resemble the *Drosophila* SOP lineage in that a progenitor cell divides twice and generates neuron, sheath and socket cells. The mechanism used for ζ lineage terminal cell fate specification appears to be different from that for the SOP lineage. In the development of *Drosophila* SOP lineage, sister cell interaction mediated by the Notch/Delta pathway

plays an important role for the specification of neuronal vs. non-neuronal cell fate (reviewed in Greenwald, 1998). However we did not observe any effect of *lin-12* on the specification of SPD vs. SPDsh fate in *C. elegans* male spicule development. Rather, the *lin-17*-mediated Wnt signaling pathway controls this fate decision. Thus the two different signaling pathways, Notch and Wnt, are used in different organisms to achieve a similar cell fate patterning in a conserved neurogenic lineage.

However, the Wnt pathway is not the sole player for spicule development. The β lineage closely resembles ζ lineage in generating the neuron and non-neuronal structural cells. Yet the Wnt pathway does not seem to be involved to the neuron/sheath fate specification derived from β lineage as revealed by other molecular markers (data not shown). In contrast to the ζ lineage, cell-cell interactions within the B lineage do play an important role during the development of the β lineage since β lineage can be easily perturbed by cell ablations or mutants of the *lin-3*/*let-23* EGF/EGFR pathway components (Chamberlin and Sternberg, 1993; 1994). Therefore, multiple signaling pathways must be utilized during male spicule development.

An HMG1/2-like protein is involved in Wnt signaling

We identified a new gene, *son-1*, which is involved in the SPD neuron vs. SPDsh fate specification in a similar fashion as *lin-17* during *C. elegans* male spicule development. *son-1* encodes an HMG1/2-like protein. A GFP reporter gene revealed that *son-1* is broadly expressed through development

and its expression is localized in cell nuclei, consistent with its being an HMG-box containing DNA binding protein.

Although the HMG1/2 class proteins are traditionally considered as architectural components of chromatin, the specific phenotypes of *son-1(sy549)* suggest that this gene may be specifically involved in Wnt signaling. First, *son-1(sy549)* causes a similar SPDsh to neuron fate transformation as does a *lin-17* hypomorph. The vulva and gonad defects observed in *son-1(sy549)* mutants, although at low penetrance, do resemble what are seen in *lin-17* mutants. Second, we observed specific postembryonic phenotypes that resemble *lin-17* mutant phenotypes from *son-1 RNAi* experiments. Third, *son-1(sy549)* enhances *lin-17(n698)* phenotypes to *lin-17* null-like but does not enhance phenotypes of other unrelated mutants such as *let-2(mn114)*. Fourth, overexpression of POP-1 gave similar phenotypes as disruption of *son-1* function. Taken together, *son-1* is involved in several Wnt-mediated developmental processes, including vulva formation, somatic gonad development, and male spicule development. Our results provide the first evidence for the specific functions of the HMG1/2 class proteins in vivo.

The HMG1/2-like protein encoded by *son-1* has several interesting features that are different from what is known about the HMG1/2 class proteins. The HMG1/2 class of proteins is classified as sequence non-specific HMG proteins in comparison to the sequence specific HMG proteins, such as TCF/LEF (Bianchi et al., 1992). The canonical HMG box found in sequence non-specific HMG proteins and the generalized HMG box found in sequence specific HMG proteins share substantial sequence homology and identical protein structure. The sequence specificity is thought to reside in the first 12

amino-terminal residues and the last 25 carboxyl-terminal residues of the HMG box. In particular the amino acids at position 7 and 12 have been suggested to be crucial for sequence specific binding (Read et al., 1994). The residues at position 7 and 12 are valine (V) or isoleucine (I) and asparagine (N) in all sequence specific HMG boxes. All presently known sequence non-specific HMG boxes have proline (P) and serine (S) instead at these two positions. The box B of SON-1 protein appears to be consistent with this finding (position 102 and 107 of the protein, Figure 4B). Interestingly, the box A of SON-1 protein has a valine (V) residue at position 7 (position 14 of the protein, Figure 4B). This raises the possibility that the *C. elegans* HMG1/2-like protein SON-1 has more specific functions than the HMG1/2-like proteins in other organisms. Furthermore, our data suggest that both HMG boxes are required in vivo for SON-1 functions, in contrast to the in vitro observations that each individual HMG box is able to recognize bent DNA structure and to interact with certain transcription factors in a similar fashion as is the whole protein (Bianchi et al., 1992; Zappavigna et al., 1996). These features of the SON-1 protein may be indicative of its specific functions.

Two classes of HMG proteins, SON-1 and POP-1, play opposite roles in Wnt signaling

The TCF/LEF class sequence specific HMG proteins have been implicated in Wnt signaling. They can either positively or negatively regulate Wnt target gene expression depending on their transcription co-factors (van de Wetering et al., 1997; Waltzer and Bienz, 1998). The *C. elegans* TCF/LEF

homologue, *pop-1*, appears to negatively regulate Wnt signaling since loss-of-function in *pop-1* causes an opposite phenotype as inactivation of the Wnt signaling during *C. elegans* embryogenesis. During postembryonic development, overexpression of POP-1 results in a spectrum of phenotypes in several *lin-17*-mediated processes as loss-of-function mutation in *lin-17* and *son-1*. Therefore, a POP-1-like activity also negatively regulates Wnt signaling during *C. elegans* postembryonic development. Both the sequence specific TCF/LEF and sequence non-specific HMG1/2 class of proteins are involved in *lin-17*-mediated Wnt signaling and they play opposite roles in regulating Wnt pathway activity.

pop-1 has been shown to act downstream of the Wnt receptor and its expression is negatively regulated by Wnt signaling (Rocheleau et al., 1997; Thorpe et al., 1997; Lin et al., 1998). Inactivation of the Wnt pathway upregulates POP-1 protein level. Here we show that *son-1* also acts in the Wnt responding cells. Therefore, both the POP-1-like protein and SON-1 act in the same cell nucleus to regulate gene transcription. The POP-like activity inhibits gene transcription, and SON-1 facilitates gene transcription (Figure 8). Considering SON-1 being an HMG1/2-like protein, we think that SON-1 could activate gene transcription in two ways. First, SON-1 could change the chromatin conformation in favor of gene expression. Second, SON-1 could physically interact with other transcription factors and help stimulate specific gene expression. The HMG1/2 class proteins have been shown to interact with POU domain proteins or HOX proteins in vitro and enhance their DNA binding and transcriptional activity (Zwilling et al., 1995; Zappavigna et al., 1996). Our data suggest that *son-1* function may not be

rate-limiting for Wnt signal transduction. And *son-1::GFP* expression does not seem to be altered in a *lin-17* mutant background. Therefore, it is possible that SON-1 acts to activate gene transcription after the inhibitory effect of POP-1 is relieved by activation of the Wnt pathway.

In *Drosophila* and *Xenopus*, Wnt responsive target genes have been identified (Bienz, 1994; Brannon and Kimelman, 1996; Carnac et al., 1997). Consensus DNA binding sites for the TCF/LEF class HMG box proteins have been found in the promoter/enhancer regions of those target genes (Florence et al., 1997; Riese et al., 1997; Brannon et al., 1997). Mutations in the *Drosophila* TCF/LEF homologue, dTCF, can abolish the maintenance of Wnt target gene expression (van de Wetering et al., 1997). However, Wnt responsive target genes have so far not been identified in *C. elegans*. Identification of such Wnt target genes is necessary to resolve the issue how SON-1 and POP-1 regulate Wnt target gene expression.

MATERIALS AND METHODS

Strains and Alleles

Routine culturing, maintenance and genetic manipulations of *C. elegans* strains were performed according to standard procedures (Brenner, 1974). N2 (Bristol) strain was used as wild type (Brenner, 1974). The following strains were used and are referenced in "*C. elegans* II" or as indicated below.

LG I: *lin-17(n671), lin-17(n698)* (Ferguson and Horvitz, 1985), *pop-1(zu189)* (Lin et al., 1995), *dpy-5(e61), hT1, hT2*

LG II: *dpy-10(e128), rol-1(e91) mom-3(or78)* (Thorpe et al., 1997), *mnC1[dpy-10(e128) unc-52(e444)]*

LG III: *dpy-17(e164), lon-1(e185), unc-36(e251), unc-32(e189), dpy-18(e364), sDf125, sDf127, sDf135, nDf16, nDf20, nDf21, pha-1(e2123ts)* (Granato et al., 1994), *hT2, qC1, dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909)*

LG IV: *dpy-20(e1282), dpy-13(e184)*

LG V: *syIs20[gpa-1::lacZ+dpy-20(+)], him-5(e1490), dpy-11(e224)*

LG X: *bar-1(sy324)* (A. Golden and P. W. S., unpublished), *bar-1(mu349)* (kindly provided by J. Whangbo and C. Kenyon), *lon-2(e678), mom-1(or10) unc-6(n102)* (Thorpe et al., 1997)

Assay for β-galactosidase activity was performed as described previously (Fire, 1992).

Anatomical and lineage analysis /Cell ablation /Photographs

Animal anatomy and cell division patterns were examined under Nomarski optics at 20°C as described by Sulston and Horvitz (1977). For laser ablation, L3 males were mounted on 5% agar pad and anesthetized with 1-3% sodium azide. Laser killing cells was performed as described (Sulston and White, 1980; Avery and Horvitz, 1987, 1989). Worms were then recovered from the agar pad and allowed to grow to adulthood at which time we examined the *gpa-1::lacZ* marker expression. Photographs of Nomarski images were taken on Kodak Tech Pan films. Photographs of β-galactosidase expression images were taken on Kodak Royal Gold ASA 25 films. GFP expression was examined using a GFP filter set and photos were taken on Fuji Provia ASA 400 film.

Isolation of son-1(sy549) allele

Using the SPD neuron marker, *gpa-1::lacZ*, an F1 clonal screen was designed to look for mutants that specifically disrupt the terminal cell fate specification during male spicule development. Standard EMS mutagenesis was employed (Brenner, 1974). The *sy549* allele was recovered from a screen of ~6,300 haploid genomes.

Cloning of son-1

son-1(sy549) was mapped to chromosome III by two-factor mapping. Three-factor mapping put *son-1(sy549)* between *lon-1* and *unc-36*. Markers used for three-factor mapping were *dpy-17(e164) unc-32(e189)*, *dpy-17(e164) unc-*

36(e251), and *lon-1(e185) unc-32(e189)*. Deficiency mapping indicated that both *sDf125* and *sDf127* delete *son-1(sy549)*, and *nDf16*, *nDf20*, *nDf21* do not delete *son-1(sy549)*. Therefore, *son-1* mutation lies between the left end point of *sDf127* and the left end point of *nDf16*. Cosmid pools or single cosmid in that region were injected into *son-1(sy549) pha-1(ts); syIs20 him-5* strain at a concentration of 25ng/μl each to test for rescue. *pha-1* is used as a germline transformation marker (Granato et al., 1994). Rescuing activity was scored primarily based on the spicule phenotype and other phenotypes were examined as well. A single cosmid C18F10 rescued *son-1(sy549)* phenotypes in all respects. Using primers LIJ#1(5'-CCCAGTTCTTACTGCTTCGCATC-3') and LIJ#2 (5'-CATGAAAACTCCACTGCAACGG-3') we PCR amplified a 3kb fragment from the 3' end of C18F10 which contains a single gene F47D12.4. This PCR fragment was able to rescue *son-1(sy549)*. *son-1* cDNA was obtained by RT-PCR using wild type N2 total RNA as template and using primers complementary to *son-1* start and stop codon regions based on GeneFinder prediction. The ~600bp products from five independent RT-PCR clones were cloned into pBluescript and sequenced. Three clones are consistent with GeneFinder prediction, the other two have an alternative splice site at the end of the first exon and results in an additional 2 amino acids without changing the rest of the protein coding. The 5'-end of the gene was confirmed by RT-PCR using SL1 primer and an internal gene specific primer (LIJ#6: 5'-ACAGGGCTCTTTTTGGTGC-3'). The 1kb genomic region of the F47D12.4 gene was PCR amplified from small numbers of *sy549* mutants (Aroian et al., 1994) and sequenced on an automated sequencing machine (Applied Biosystems). Four independent PCR clones showed the same

nucleotide change of g→a at nucleotide position 228 (two plus strand, two minus strand). This change results in the amino acid change of aspartic acid to asparagine at amino acid residue 72.

Molecular Biology

son-1-GFP: the transcriptional fusion GFP constructs contain either 1kb or 4.5kb upstream genomic sequence and the first 64 amino acids of *son-1* protein (at the EcoRV site). The upstream sequence was PCR amplified from cosmid C18F10, the coding region was derived from the 3kb rescuing fragment. The translational fusion GFP constructs (pLJ33/pJ35) contain PCR fragment of either 1kb or 3.2kb upstream genomic sequence and full length but the last 6 amino acids of *son-1* protein cloned into GFP vectors from the Fire lab.

hs-son-1: a 1.4kb EcoRI fragment of *son-1* genomic sequence was cloned into vectors containing heat shock promoter *hsp16-2* or *hsp16-48* to generate pLJ25 and pLJ26, respectively.

son-1 deletion constructs: To create *son-1* deletion constructs, we started from the 3kb rescuing fragment. The 3kb fragment was cloned into pCRII vector (Invitrogen; pLJ13). For the box A deletion construct (pLJ41), a SmaI/EcoRV fragment was removed and re-ligated which deleted amino acids 7 to 76. For the box B deletion construct (pLJ40), sequences between BamHI and SpeI was deleted, thereby removing amino acids 99 to 162. For the box A only construct (pLJ39), a stop codon was generated after the first 98 amino acids. All constructs still contain 1kb upstream and 1kb downstream genomic sequence of *son-1*.

Generation of transgenic animals

Transgenic lines carrying extrachromosomal array of testing plasmids were generated using standard germline injection and DNA transformation method (Mello and Fire, 1995). GFP constructs were injected into *pha-1(ts); him-5(e1490)* strain at a concentration of 20ng/μl together with *pha-1* rescuing plasmid pBX-1 at 100ng/μl. Heat shock constructs and *son-1* deletion constructs were injected into *pha-1(ts); syIs20 him-5* strain. Injection concentrations for heat shock constructs containing *son-1* genomic DNA, *lin-44* cDNA, and *pop-1* cDNA are 50ng/μl, 100ng/μl, and 20ng/μl or 100ng/μl respectively. *son-1* deletion constructs were injected at 50ng/μl and 100ng/μl. To test the ability to rescue *son-1* phenotypes, transgenic lines were further crossed into *son-1(sy549) pha-1(ts); syIs20 him-5* strain.

RNA interference experiments

Double strand RNA was synthesized as described (Fire et al., 1998) using full length *son-1* cDNA as template. Estimated 40-50ng/μl dsRNA was injected into *syIs20 him-5* hermaphrodites. Eggs laid between 3 to 51 hours after injection were collected at 12-hour intervals. Phenotypes of F1s were analyzed in detail.

Heat shock experiments

Worms were staged under Nomarski optics and heat shocked in a 33°C water bath for 30, 45 or 60 minutes.

ACKNOWLEDGEMENTS

We thank Jane Mendel for the *syIs20 him-5* strain, Mike Herman for the *lin-17(n698)* allele and the *hs-lin44* constructs, Helen Stewart for the *sDf* strains and *sDf* break point mapping data, Rueyling Lin for the *hs-pop-1* construct, Jennifer Whangbo for the *bar-1(mu349)* allele, the CGC for strains and the Sanger center for cosmids and sequencing information. We thank members of the Sternberg lab for discussion and comments on the manuscript. This work was supported by a USPHS grant HD23690 to P.W. S. and by the Howard Hughes Medical Institute, with which P. W. S. is an investigator.

REFERENCES

Aroian, R. V., Lesa, G. M., and Sternberg, P. W. (1994). Mutations in the *Caenorhabditis elegans let-23* EGFR-like gene define elements important for cell-type specificity and function. *EMBO J.* **13**: 360-366.

Bianchi, M. E., Falciola, L., Ferrari, S., and Lilley, D. M. J. (1992). The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. *EMBO J.* **11**: 1055-1063.

Brannon, M., and Kimelman, D. (1996). Activation of *siamois* by the Wnt pathway. *Dev. Biol.* **180**: 344-347.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., Kimelman, D. (1997). A β-catenin/XTcf-3 complex binds to the *siamois* promoter to regulate dorsal axis specification in *Xenopus*. *Genes & Dev.* **11**: 2359-2370.

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

Burdine, R. D., Branda, C. S., and Stern, M. J. (1998). *egl-17(fgf)* expression coordinates the attraction of the migrating sex myoblasts with vulval induction *C. elegans*. *Development* **125**: 1083-1093.

Bustin, M., and Reeves, R. (1996). High mobility group chromosomal proteins: Architectural components that facilitate chromatin function. *Progress in Nucleic Acid Res. and Molec. Biol.* **54**: 35-100.

Cadigan, K. M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Gene & Dev.* **11**: 3286-3305.

Carnac, G., Kodjabachian, L., Gurdon, J. B., and Lemaire, P. (1996). The homeobox gene *siamois* is a target of the Wnt dorsalization pathway and

triggers organizer activity in the absence of mesoderm. *Development* **122**: 3055-3065.

Chamberlin, H. M., and Sternberg, P. W. (1993). Multiple cell interactions are required for fate specification during male spicule development in *Caenorhaebditis elegans.*. *Development* **118**: 297-324.

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

Chamberlin, H. M., and Sternberg, P. W. (1995). Mutations in the *Caenorhabditis elegans* gene *vab-3* reveal distinct roles in fate specification and unequal cytokinesis in an asymmetric cell division. *Devel. Biol.* **170**: 679-689.

Collet, J., Spike, C. A., Lundquist, E. A., Shaw, J. E., and Herman, R. K. (1998). Analysis of *osm-6*, a gene that affects sensory cilium structure and sensory neuron function in *Caenorhabditis elegans*. *Genetics* **148**: 187-200.

Eisenmann, D. M., Maloof, J. N., Simske, J. S., Kenyon, C., and Kim, S. K. (1998). The β-catenin homolog BAR-1 and LET-60 RAS coordinately regulate the hox gene *lin-39* during *Caenorhabditis elegans* vulval development. *Development* **125**: 3667-3680.

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

Ferguson, E. L., Sternberg, P. W., and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**: 259-267.

Fire, A. (1992). Histochemical techniques for locating *Escherichia coli* beta-galactosidase activity in transgenic organisms. *Genet. Anal.* **9**: 151-158.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806-811.

Florence, B., Guichet, A., Ephrussi, A., and Laughon, A. (1997). Ftz-F1 is a cofactor in Ftz activation of the *Drosophila engrailed* gene. *Development* **124**: 839-847.

Granato, M., Schnabel, H, and Schnabel, R. (1994). *pha-1*, a selectable marker for gene-transfer in *C. elegans*. *Nucl. Acid Res.* **22**: 1762-1732.

Hartenstein, V., Posakony, J. W. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. *Dev. Biol.* **142**: 13-30.

Herman, M. A., and Horvitz, H. R. (1994). The *Caenorhabditis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. *Development* **120**: 1035-1047.

Herman, M. A., Vassilieva, L. L., Horvitz, H. R., Shaw, J. E., and Herman, R. K. (1995). The *C. elegans* gene *lin-44*, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. *Cell* **83**: 101-110.

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

Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T., and Ptashne, M. (1994). An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371**: 175-179.

Lin, R. L., Thompson, S., and Priess, J. R. (1995). *pop-1* encodes an HMG box protein reuired for the specification of a mesoderm precursor in early C. elegans embryos. *Cell* **83**: 599-609.

Lin, R. L., Hill, R. J., and Priess, J. R. (1998). POP-1 and anterior-posterior fate decisions in *C. elegans* embryo. *Cell* **92**: 229-239.

Mello, C. C., and Fire, A. (1995). Methods in Cell Biology: *C. elegans*. (New York: Academic Press).

Meneely, P. M., and Wood, W. B. (1987). Genetic analysis of X-chromosome dosage compensation in *Caenorhabditis-elegans*. *Genetics* **117**: 25-41.

Moon, R. T., Brown, J. D., and Torres, M. (1997). Wnts modulate cell fate and behavior during vertebrate development. *TIG* **13**: 157-162.

Parks, A. L., Muskavitch, M. A. (1993). *Delta* function is required for bristle organ determination and morphogenesis in *Drosophila*. *Dev. Biol.* **157**: 484-496.

Read, C. M., Cary, P. D., Preston, N. S., Lenicek-Allen, M., and Crane-Robinson, C. (1994). The DNA-sequence specificity of HMG boxes lies in the minor wing of the structure*EMBO J.* **13**: 5639-5646.

Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J. R. (1997). *C. elegans* II. (eds). Cold Spring Harbor Laboratory Press.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from *wingless* and *decapentaplegic*. *Cell* **88**: 777-787.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.-H., Ali, M., Priess, J. R., and Mello, C. C. (1997) Wnt signaling and an

APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**: 707-716.

Roehl, H., Kimble, J. (1993). Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. *Nature* **364**: 632-635.

Sawa, H., Lobel, L., and Horvitz, H. R. (1996). The *Caenorhabditis elegans* gene *lin-17*, which is required for certain asymmetric cell divisions, encodes a putative protein similar to seven-transmembrane protein similar to the *Drosophila* Frizzle protein. *Genes Devel.* **10**: 2189-2197.

Sibley, M. H., Graham, P. L., Vonmende, N., and Kramer, J. M. (1994). Mutations in the alpha-2(IV) basement-membrane collagen gene of *Caenorhabditis elegans* produce phenotypes of differing severities. *EMBO J.* **13**: 3278-3285.

Sternberg, P. W., and Horvitz, H. R. (1988). *lin-17* mutations of *Caenorhabditis elegans* disrupt certain asymmetric cell divisions. *Dev. Biol.* **130**: 67-73.

Sulston, J. E., and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Devel. Biol.* **56**: 110-156.

Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Devel. Biol.* **78**: 577-597.

Sulston, J. E., Schierenberg, E., White, J. G., Thomson, J. N. (1983). The embryonic-cell lineage of the nematode *Caenorhabditis elegans*. *Devel. Biol.* **100**: 64-119.

Thorpe, C., Schlesinger, A., Carter J. C., and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**: 695-705.

van de Wetering, M. Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* **88**: 789-799.

Waltzer, L., and Bienz, M. (1998). *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signaling. *Nature* **395**: 521-525.

FIGURES

Figure 1. The B lineage.

The B cell division pattern from 1-cell to 10-cell stage and the neurogenic sublineages β and ζ are shown (adapted from Sulston et al. 1980; Chamberlin and Sternberg, 1993). sh: sheath cell; so: socket cell. SPV neuron is derived from β sublineage and SPD neuron from ζ sublineage. SPD neuron and SPD sheath cell are sister cells derived from ζ.pa.

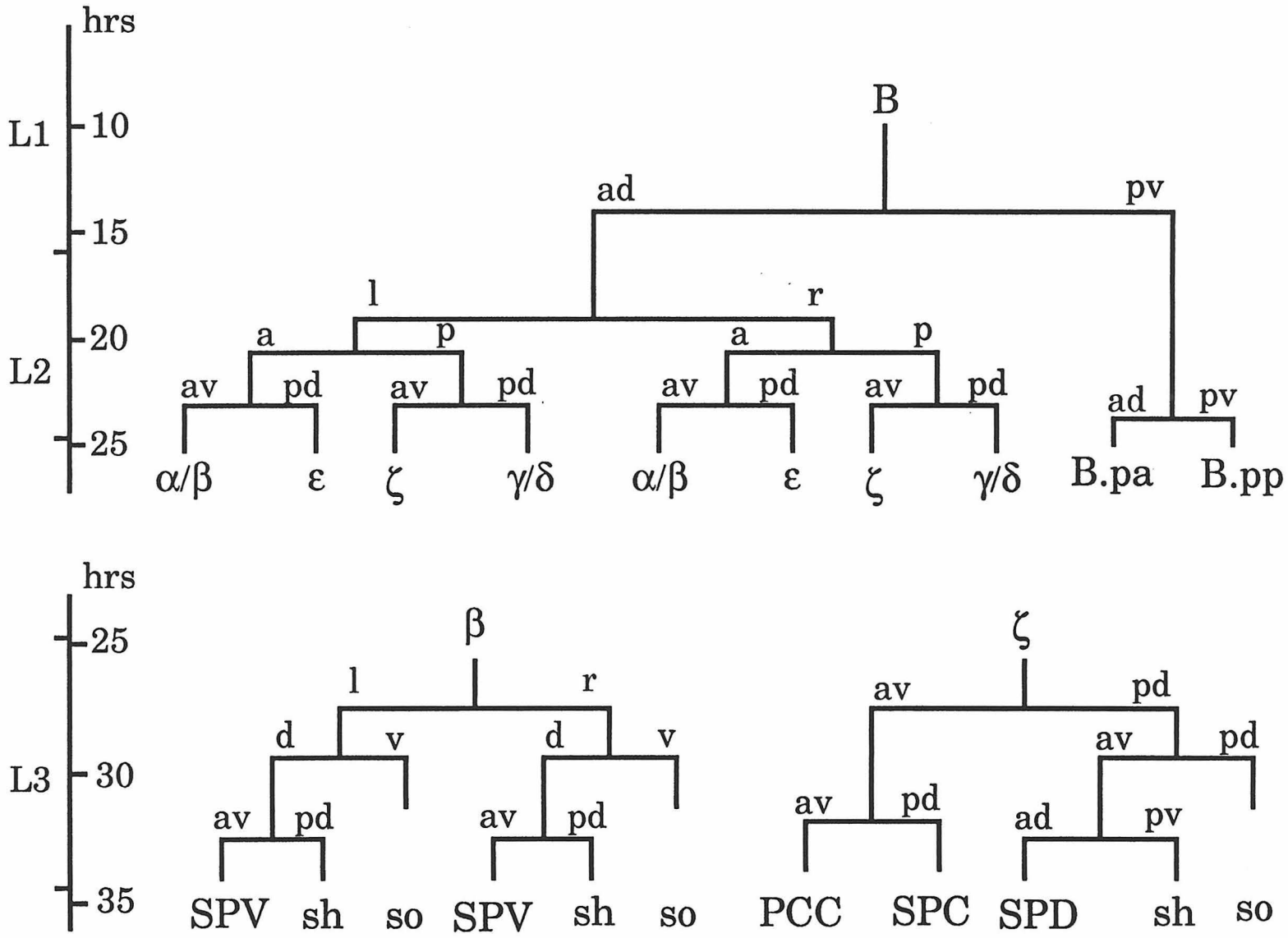
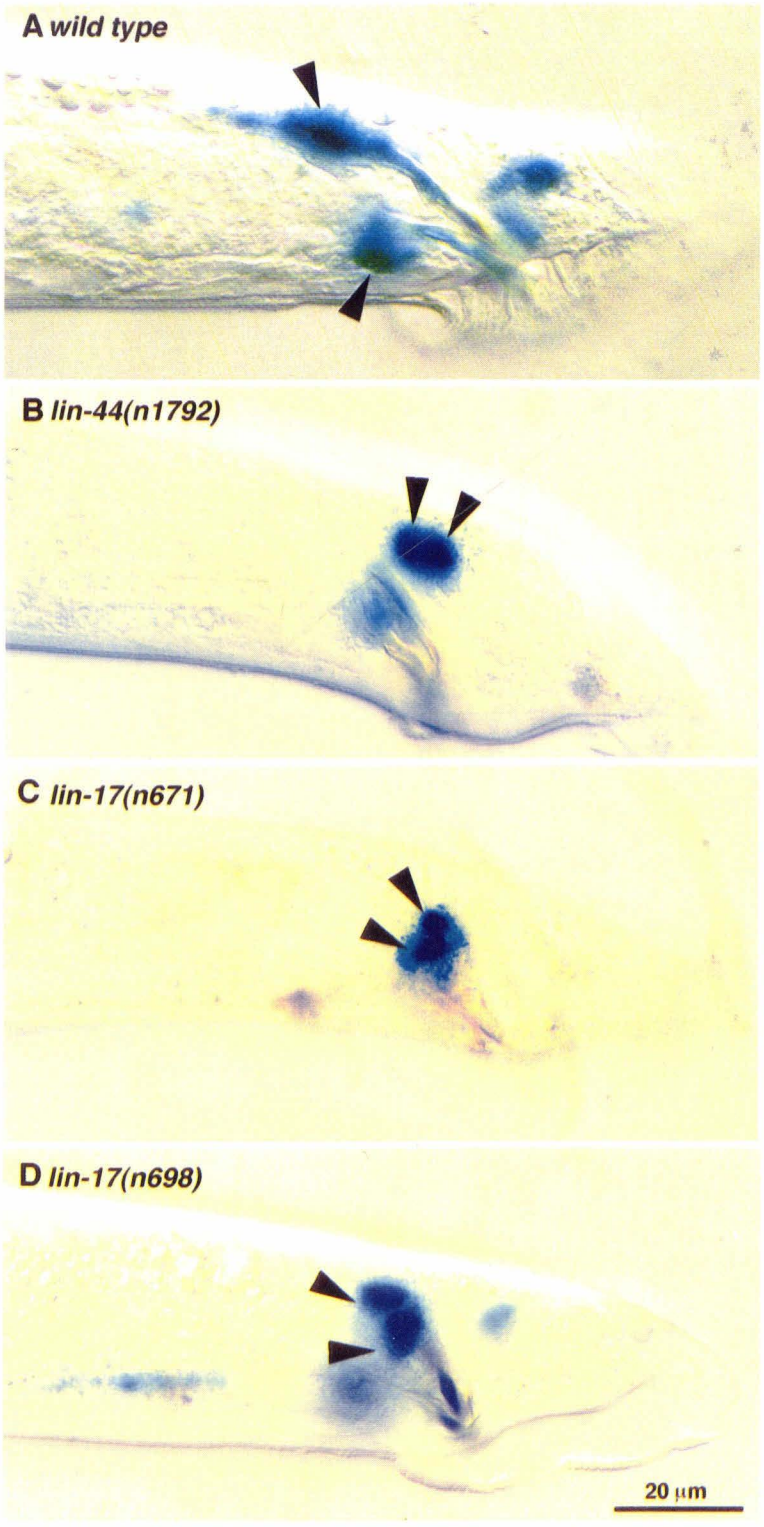

hrs
L1
10
15
L2
20
25
B
ad
pv
l
r
a
p
a
p
av
pd
av
pd
av
pd
av
pd
ad
pv
α/β
ε
ζ
γ/δ
α/β
ε
ζ
γ/δ
B.pa
B.pp
hrs
L3
25
30
35
β
ζ
l
r
av
pd
d
v
d
v
av
pd
av
pd
av
pd
av
pd
ad
pv
SPV
sh
so
SPV
sh
so
PCC
SPC
SPD
sh
so

Figure 2. SPD neuron marker is expressed in 2-cell per spicule in Wnt mutants, *lin-44* and *lin-17*, and *son-1(sy549)*.

(A) In wild-type males, the *gpa-1::lacZ* marker is expressed in one cell per spicule. Cell ablation experiment identifies this cell being SPD neuron. Both spicules are shown on this focal plane.

(B-D). *gpa-1::lacZ* is expressed in two cells per spicule in *lin-44* and *lin-17* mutant males. (B). *lin-44(n1792)*; (C) *lin-17(n671)*; (D). *lin-17(n698)*, early B lineage defect is rare in this weak allele and ~80% males show a two cells per spicule marker expression pattern. Lateral view, only one spicule is in focus.

(E-H). *gpa-1::lacZ* marker is expressed in two cells per spicule in *son-1(sy549)* mutants. Cell ablation experiment indicated that *son-1(sy549)* confers a SPD sheath to neuron fate transformation. (E). *sy549* intact animal, lateral view showing the left spicule; (F). When the precursor of SPD neuron and SPD sheath is ablated, no expression is observed in the spicule region; (G). When SPD neuron is ablated, SPD marker is expressed in one cell per spicule. (H). When SPD sheath is ablated, SPD marker is expressed in one cell per spicule. Scale bar indicates 20μm.


A wild type
B lin-44(n1792)
C lin-17(n671)
D lin-17(n698)
20 μm

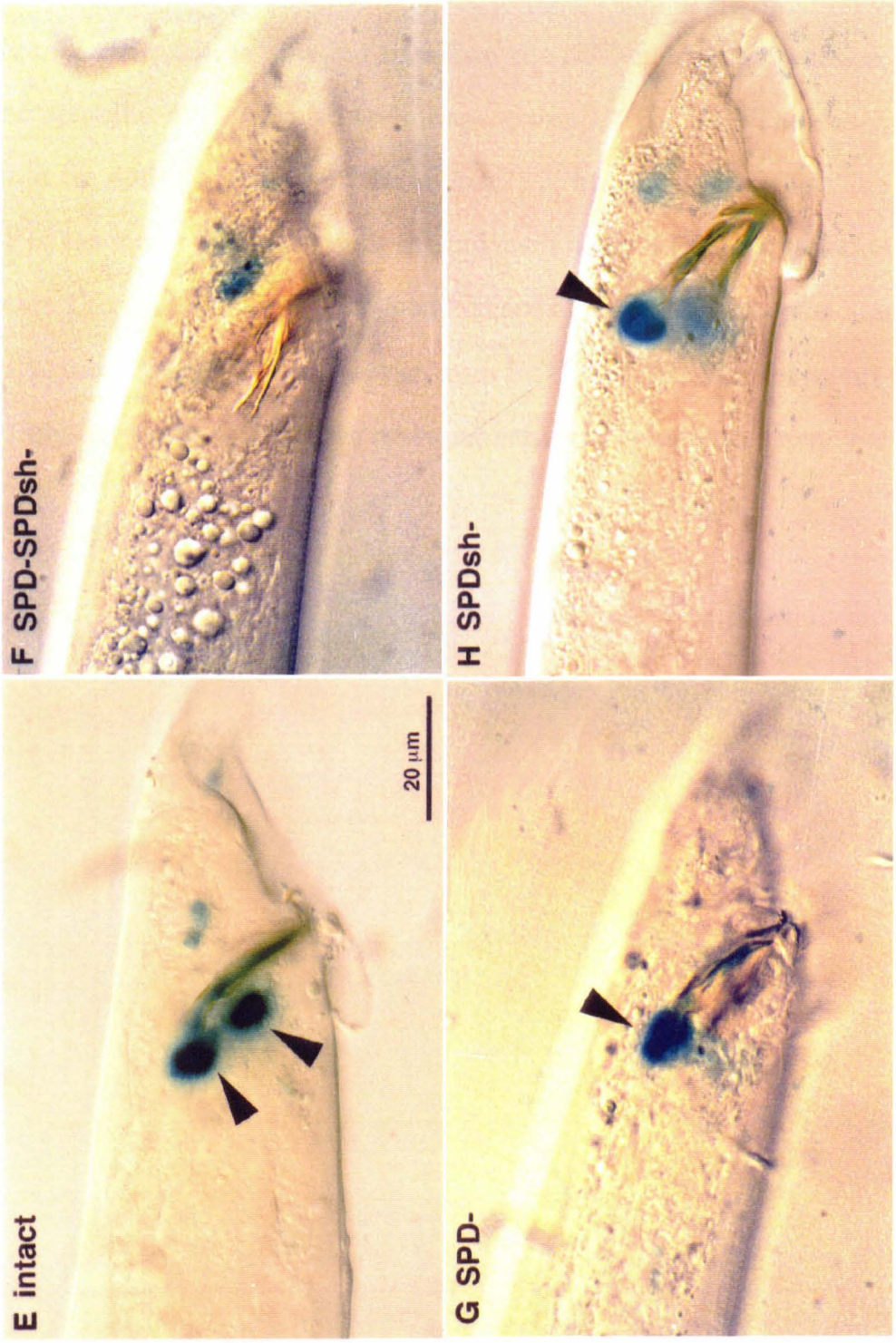

E intact
F SPD-SPDsh-
G SPD-
H SPDsh-
20 μm

Figure 3: Mapping and cloning of *son-1*

The *son-1* mutation is mapped based on the SPD marker expression pattern in the spicules. It is mapped to linkage group III (LGIII). The *son-1* locus is deleted by deficiencies *sDf125* and *sDf127*, but not by *nDf16*. The cosmid C18F10 rescued *son-1* mutation, the adjacent cosmid F47D12 failed to rescue. Primers 1kb upstream and 1kb downstream of the F47D12.4 gene amplified a 3kb fragment from C18F10 but not from F47D12. This 3kb fragment rescued *son-1* phenotypes. (+) indicates rescuing activity, (-) indicates non-rescue.

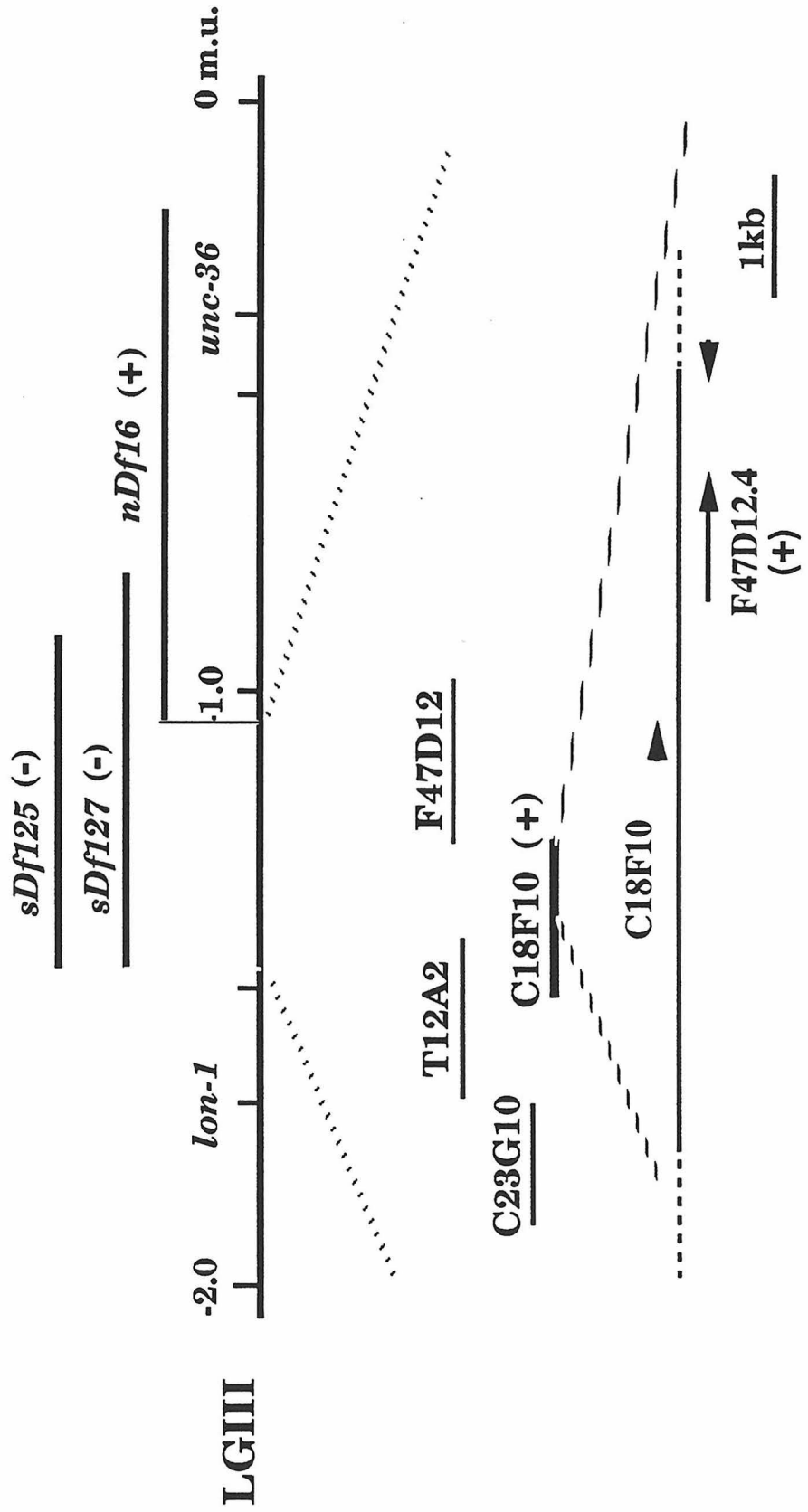
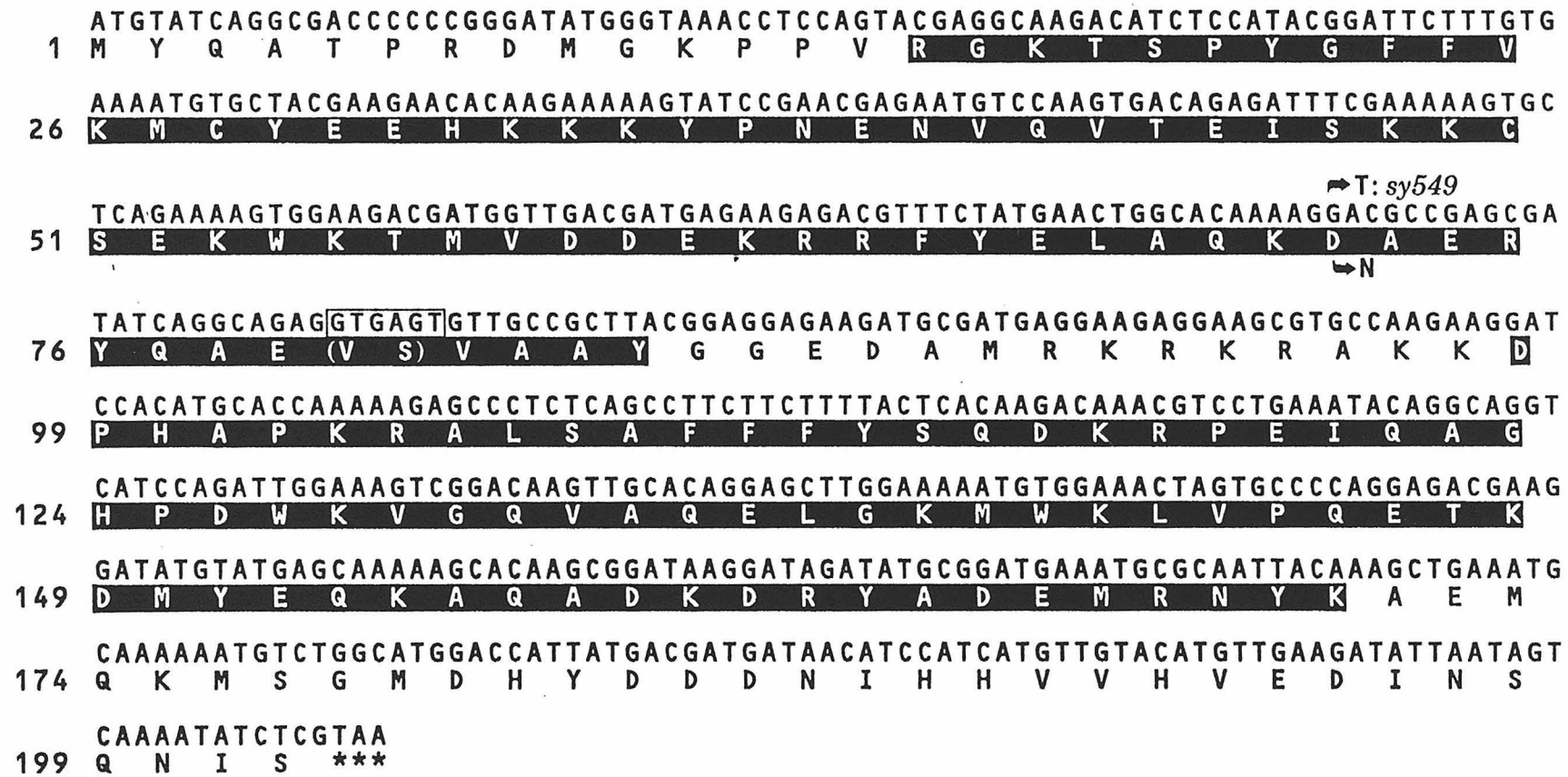

LGIII
-2.0
lon-1
-1.0
unc-36
0 m.u.
sDf125 (-)
sDf127 (-)
nDf16 (+)
T12A2
F47D12
C23G10
C18F10 (+)
C18F10
F47D12.4
(+)
1kb

Figure 4: Sequence of the *son-1* gene

(A). *son-1* cDNA sequence and deduced amino acid sequence. The boxed sequence is derived from alternative splicing at the end of exon 1, which leads to additional two amino acids as indicated in parenthesis. This alternative splicing does not change the overall protein structure. Shaded region indicates HMG box A and box B. The stop codon is indicated by three asterisks. Molecular lesion of the *sy549* allele is indicated by arrow.

(B). Comparison of the HMG box A and box B sequences between SON-1 and HMG1-like proteins of *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*. Consensus resides between SON-1 and other HMG1-like proteins are shaded. Asterisk indicates the unusual valine residue found in the box A of SON-1 protein.

A.


ATGTATCAGGCGACCCCCCGGGATATGGGTAAACCTCCAGTACGAGGCAAGACATCTCCATACGGATTCTTTGTG
1 M Y Q A T P R D M G K P P V R G K T S P Y G F F V
AAAATGTGCTACGAAGAACACAAGAAAAAGTATCCGAACGAGAATGTCCAAGTGACAGAGATTTCGAAAAAGTGC
26 K M C Y E E H K K K Y P N E N V Q V T E I S K K C
T: sy549
TCAGAAAAGTGGAAGACGATGGTTGACGATGAGAAGAGACGTTTCTATGAACTGGCACAAAAGGACGCCGAGCGA
51 S E K W K T M V D D E K R R F Y E L A Q K D A E R
N
TATCAGGCAGAGGTGAGTGTTGCCGCTTACGGAGGAGAAGATGCGATGAGGAAGAGGAAGCGTGCCAAGAAGGAT
76 Y Q A E (V S) V A A Y G G E D A M R K R K R A K K D
CCACATGCACCAAAAAGAGCCCTCTCAGCCTTCTTCTTTTACTCACAAGACAAACGTCCTGAAATACAGGCAGGT
99 P H A P K R A L S A F F F Y S Q D K R P E I Q A G
CATCCAGATTGGAAAGTCGGACAAGTTGCACAGGAGCTTGGAAAAATGTGGAAACTAGTGCCCCAGGAGACGAAG
124 H P D W K V G Q V A Q E L G K M W K L V P Q E T K
GATATGTATGAGCAAAAAGCACAAGCGGATAAGGATAGATATGCGGATGAAATGCGCAATTACAAAGCTGAAATG
149 D M Y E Q K A Q A D K D R Y A D E M R N Y K A E M
CAAAAAATGTCTGGCATGGACCATTATGACGATGATAACATCCATCATGTTGTACATGTTGAAGATATTAATAGT
174 Q K M S G M D H Y D D D N I H H V V H V E D I N S
CAAAATATCTCGTAA
199 Q N I S ***

B.

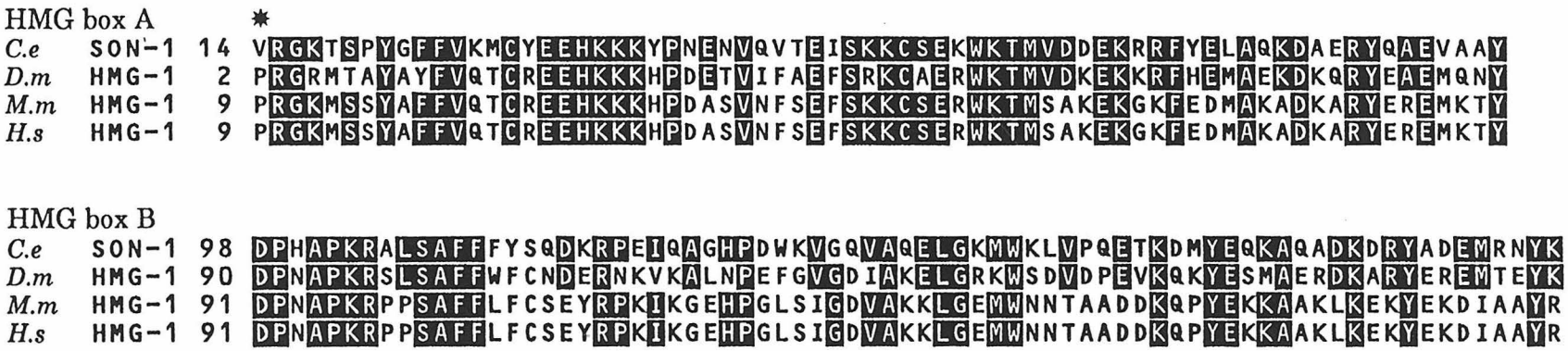

HMG box A
C.e SON-1 14 VRGKTSPYGFFVKMCYEEHKKKYPNENVQVTEISKKCSEKWKTMVDDEKRRFYELAQKDAERYQAEVAAY
D.m HMG-1 2 PRGRMTAYAYFVQTCREEHKKKHPDETVIFAEFSRKCAERWKTMVDKEKKRFHEMAEKDKQRYEAEMQNY
M.m HMG-1 9 PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTY
H.s HMG-1 9 PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTY
HMG box B
C.e SON-1 98 DPHAPKRALSAFFFYSQDKRPEIQAGHPDWKVGQVAQELGKMWKLVPQETKDMYEQKAQADKDRYADEMRNYK
D.m HMG-1 90 DPNAPKRSLSAFFWFCNDERNKVKALNPEFGVGDIAKELGRKWSDVDPEVKQKYESMAERDKARYEREMTEYK
M.m HMG-1 91 DPNAPKRPPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYR
H.s HMG-1 91 DPNAPKRPPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYR

Figure 5. A GFP reporter construct reveals that *son-1* is expressed in all somatic cell nuclei through development.

(A). An L3 male.

(B). An L1 hermaphrodite.

(C). Male tail at L4 molt.

Scale bar indicates 20μm.

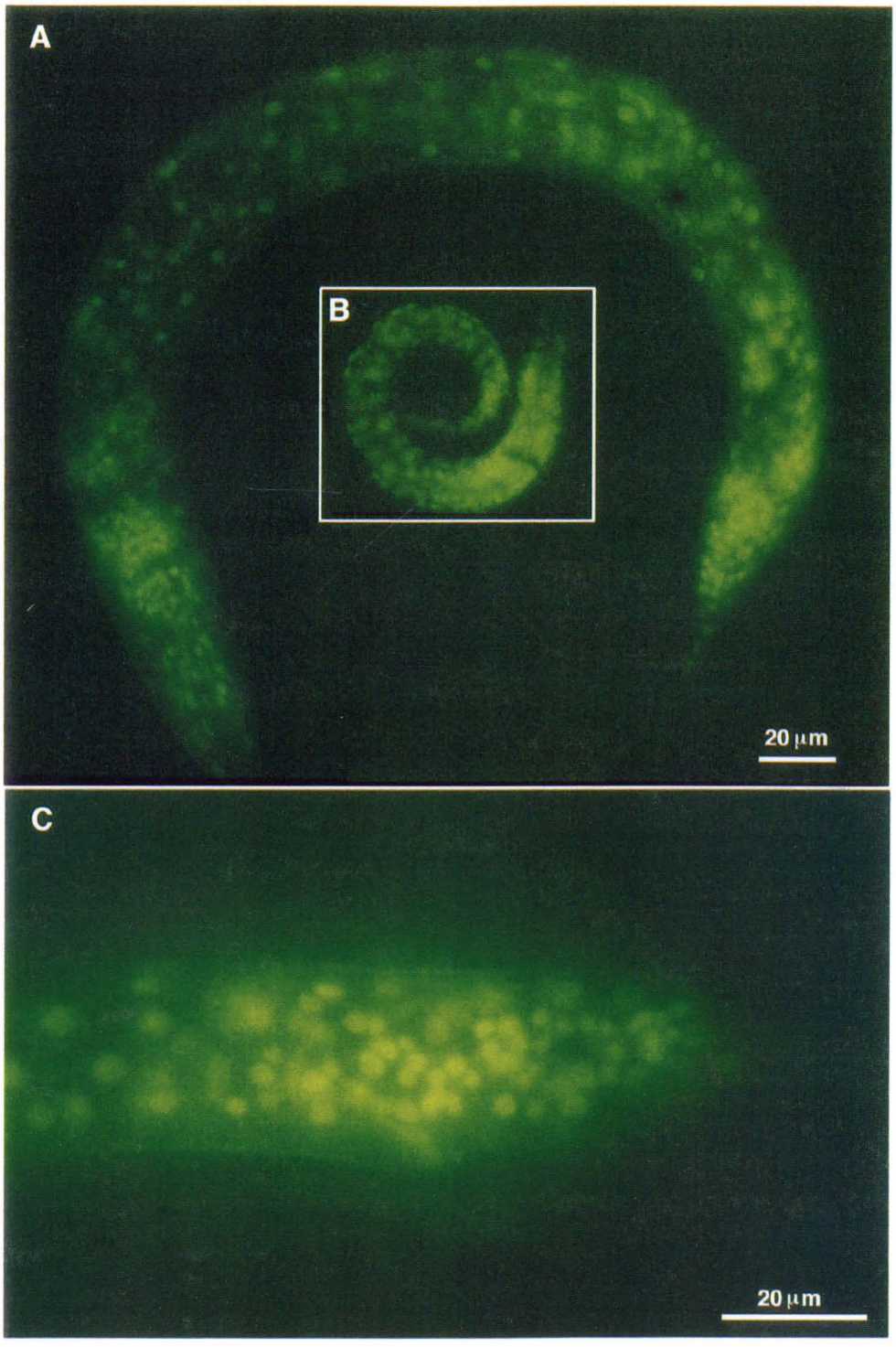

A
B
20 μm
C
20 μm

Figure 6: Disruption of *son-1* function by *RNAi* and overexpression of POP-1 result in the same spectrum of phenotypes.

(A-C): phenotypes observed from *son-1 RNAi* experiment; (D-F): phenotypes observed in POP-1 overexpression experiment. (A, D): hermaphrodite gonad, showing abnormally short gonad arm, arrow indicates meiosis at the distal end of the gonad arm. (B, E): male gonad, white arrowhead indicates ectopic germline meiosis. (C, F): *gpa-1::lacZ* expression in the spicule region, black arrowhead indicates that two cells per spicule express the marker. (C). all four blue cells can be seen on this focal plane, two from the left spicule and two from the right one. Cells from the same side are indicated by linkage of two arrowheads. (F). lateral view, showing only the left spicule.

Scale bar indicates 20μm.

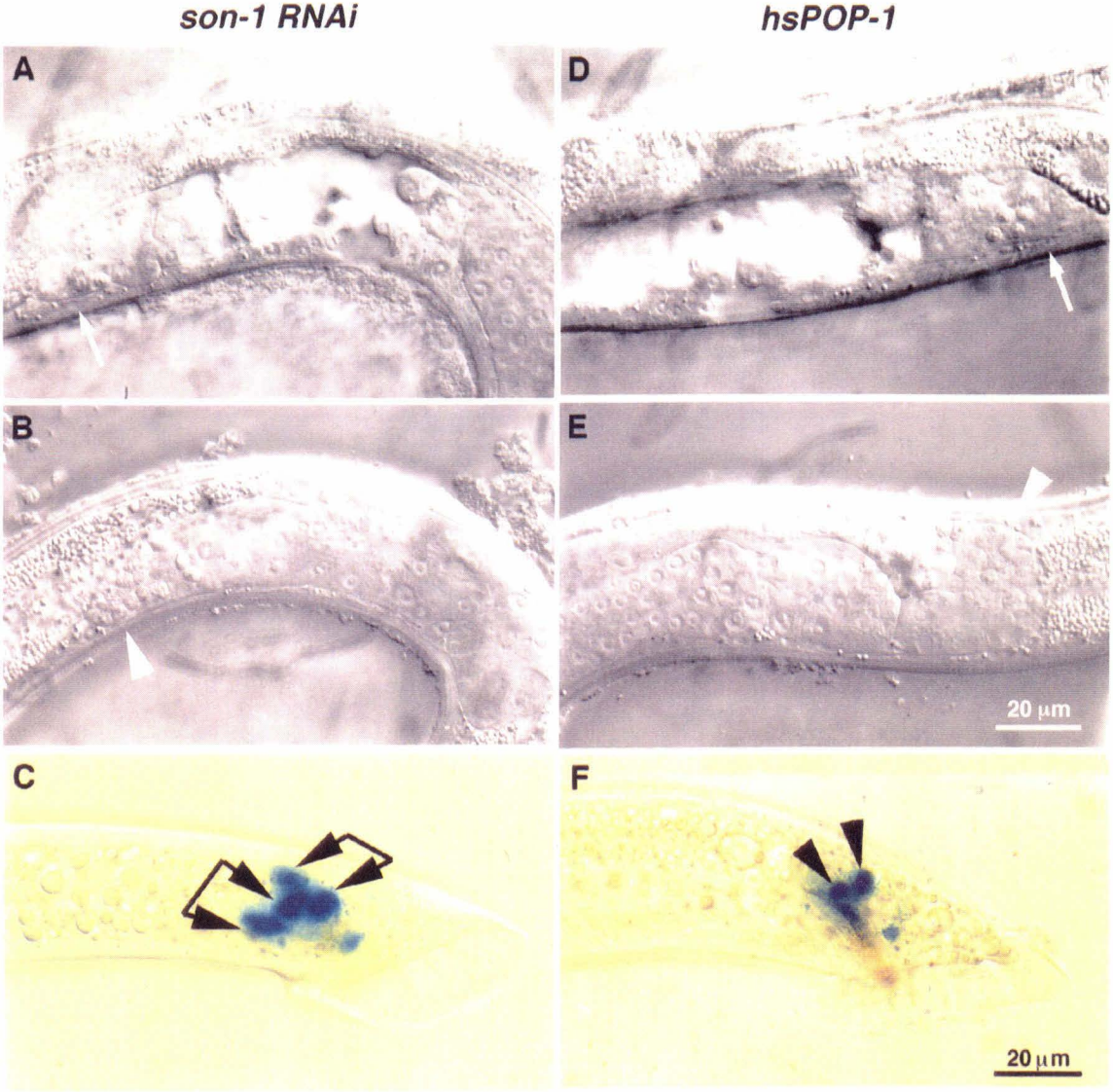

son-1 RNAi
hsPOP-1
A
D
B
E
20 µm
C
F
20 µm

Figure 7: Structure/function analysis of HMG boxes.

HMG boxes are indicated as open box and labeled. Bold line indicates the acidic tail. All constructs contain additional 1kb upstream and 1kb downstream genomic sequences.

a: Constructs were tested in wild-type background for any dominant negative or gain-of-function effect.

b: Constructs were tested in *son-1(sy549)* mutant background for any rescuing activity.

+: positive for tested activity; -: negative for tested activity.

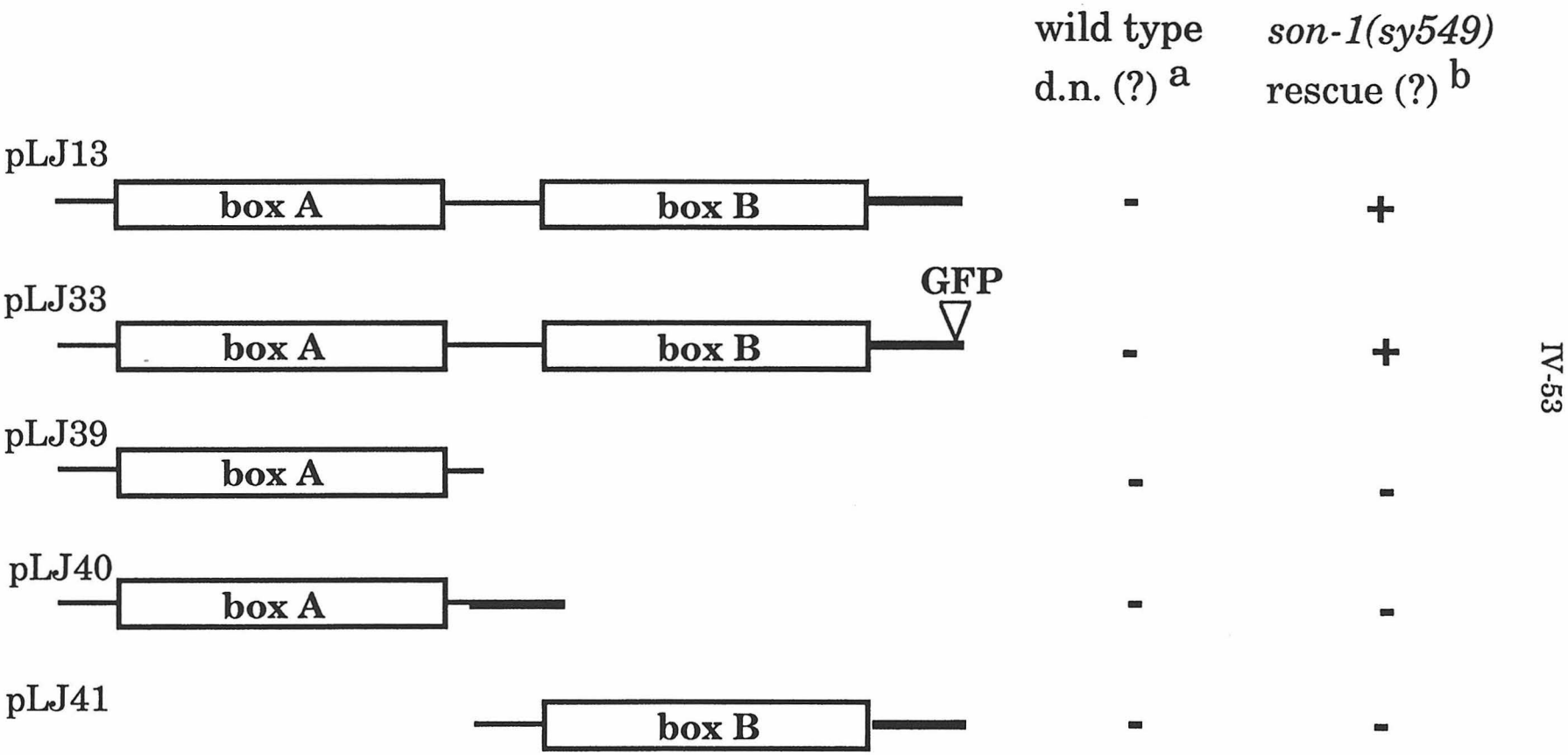
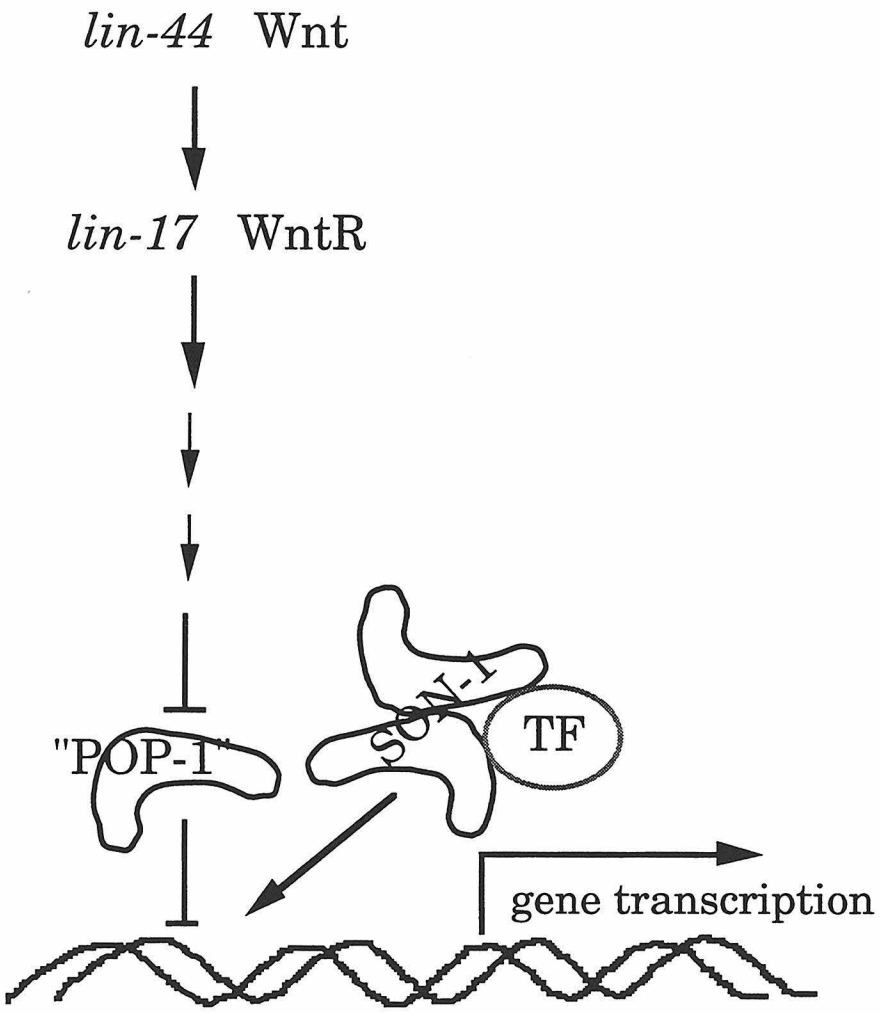

wild type
d.n. (?) a
son-1(sy549)
rescue (?) b
pLJ13
box A
box B
-
+
pLJ33
GFP
box A
box B
-
+
pLJ39
box A
-
-
pLJ40
box A
-
-
pLJ41
box B
-
-

Figure 8. Model for SON-1 function.

SON-1 and a POP-1-like protein antagonistically regulate gene expression in Wnt responding cells. The POP-1-like activity inhibits gene transcription, while SON-1 facilitates gene transcription. SON-1 might facilitate gene transcription after the inhibitory effect of the POP-1-like activity is relieved upon activation of the Wnt pathway. SON-1 could change the chromatin conformation in favor of gene expression. Alternatively, SON-1 could interact with other transcription factors to help stimulate gene expression. "POP-1" denotes a POP-1-like protein, TF denotes transcription factor.


lin-44 Wnt
lin-17 WntR
"POP-1"
SON-1
TF
gene transcription

TABLES

Table 1. Both *lin-17(n698)* and *son-1(sy549)* confer a SPD sheath to neuron fate transformation.

genotype	*lin-17(n698)*		*son-1(sy549)*	
cell(s) ablated	n [a]	marker expression [b]	n [a]	marker expression [b]
intact	15/17	**2**	39/52	**2**
β	5/6	**2**	4/6	**2**
ζl/r	15/16	**0**	12/15	**0**
ζl/r a	13/15	**2**	5/5	**2**
ζl/r p	5/5	**0**	6/9	**0**
ζl/r pp	4/7	**2**	1/2	**2**
ζl/r pa (SPD+SPDsh)	11/11	**0**	12/14	**0**
ζl/r paa (SPD)	10/13	**1**	13/16	**1**
ζl/r pap (SPDsh)	13/21	**1**	6/7	**1**

a: Numbers of animals operated and scored.

b: Numbers indicate number of cell(s) per spicule expressing the *gpa-1::lacZ* marker.

Cell(s) were ablated at L3 stage and operated animals were recovered to allow reaching adulthood. Adult males were then examined for *gpa-1::lacZ* marker expression.

Table 2: *son-1(sy549)* enhances *lin-17(n698)* phenotypes to *lin-17* null-like.

genotype		phenotypes			
lin-17	*son-1*	bi-vulva[a]	hermaphrodite gonad [b]	P12.p→P11.p [c]	abnormal phasmids [d]
+	*sy549*	2% (n=108)	1% (n=108)	0 (n=100)	4% (n=53)
n698	+	2% (n=46)	8% (n=46)	0 (n=100)	73% [f] (n=346)
n698	*sy549*	58% (n=98)	28% (n=101)	0 (n=100)	98% (n=57)
n671	+	64% (n=66)	23% (n=66)	29% [e] (n=41)	95% [f] (n=400)
n671	*sy549*	64% (n=76)	20% (n=69)	22% (n=92)	N.D.

Table 2:

a: Bi-vulva phenotype is defined as two vulval invaginations at mid to late L4 stage.

b: Hermaphrodite gonad defect is defined as abnormal gonad morphology, single gonadal arm, and ectopic meiosis at L4 stage.

c: P11/P12 phenotype is scored by the nuclear morphologies and positions of P11.p and P12.pa cells at L4 stage.

d: Worms of L3 and L4 stages were tested by DiO filling of amphids and phasmids as described by Herman and Horvitz (1994). Animals that showed positive for amphid filling and negative for phasmid filling were scored as phasmid defective.

e: Data from Jiang and Sternberg (1998).

f: Data from Sawa et al. (1996).

N.D.: not determined.

Chapter V

Socket cells mediate spicule morphogenesis in *Caenorhabditis elegans* males

(prepared for submission)

ABSTRACT

C. elegans **male spicule morphogenesis requires the coordinated cellular behaviors of several types of cells. We found that the spicule neurons and sheath cells, although important for spicule function, are dispensable for spicule morphology. In contrast, the spicule socket cells are essential for both spicule elongation and secretion of spicule cuticle. The socket cells are not only necessary but also sufficient to produce spicule cuticle. This functional aspect of socket cells is genetically separable from their function in mediating spicule elongation: elongated spicules lacking spicule cuticle can be formed. During spicule morphogenesis, the expression of an *egl-17::GFP* reporter gene is found in the spicule socket cells appears to be regulated in the socket cells. Mutants defective in TGF-β signaling display a crumpled spicules phenotype as a result of failure of socket cell movement during spicule morphogenesis. These observations suggest that that both the FGF and the TGF-β signaling pathways might be involved in spicule elongation.**

INTRODUCTION

Morphogenesis and organogenesis is a developmental process that requires coordination of a population of cells. To form a certain structure, specialized cells have to be generated at the correct positions, possess specific morphogenetic properties, including cell migration, contact guidance, and cell adhesion, and exert specific functions. This process involves the specification of terminal cell fates, which in turn regulates cellular behaviors and cell functions. Although cell fate specification is the initial and the most important aspect of organogenesis, the subsequent cellular behaviors and cell functions are also essential for morphogenic events and organogenesis.

We have been using the genetically tractable model system *Caenorhabditis elegans* to study organogenesis. Morphogenesis of *C. elegans* male spicules provides a good opportunity for this study. Spicules are specialized copulatory structures in nematode male tails. In *C. elegans* each male has a pair of spicules, which are used during the mating process to protrude into the hermaphrodite vulva, anchor the male tail to the vulva, and facilitate sperm transfer. Each spicule is composed of two neurons, two sheath cells, and four socket cells (Sulston et al., 1980; Figure 1A). The two neurons, SPD and SPV, send their processes through the spicule to a pore at the tip of the spicule, which may allow the ciliated sensory endings of the two neurons to sense the environment (Sulston et al., 1980). Therefore, they are thought to be chemosensory neurons. The SPD and SPV neurons are responsible for different steps in mating behavior: SPD is required for spicule insertion into the vulva while SPV is responsible for proper sperm transfer

(Liu and Sternberg, 1995). The two sheath cells fuse with each other and enwrap the processes of the spicule neurons. The four socket cells also fuse together and form the outer layer of the spicule. A layer of sclerotized refractile cuticle material is laid down outside of each spicule, which we call spicule cuticle.

The whole spicule structure is generated from a single male specific blast cell, the B cell. At the end of second larval (L2) stage, B.a generates eight progeny which assume six distinct fates, designated as α, β, γ, δ, ε (2), and ζ (2) (Sulston et al., 1980; Chamberlin and Sternberg, 1993; Figure 1B). During the L3 stage, these cells continue to divide to generate spicule neurons, sheath cells, socket cells, and connective tissues. All the spicule neurons and sheath cells are derived from the β and ζ sublineages. The socket cells are generated by α, β, ζ, and γ sublineages, one socket cell from each lineage. Other sublineages give rise to part of the proctodeum which is the junction between the spicules and other structures in the tail region (i.e., alimentary tract, genital tract, sex muscles, etc.). All the cells are born by the end of L3 stage, yet spicule morphogenesis does not start until mid-L4 stage. During mid-L4 stage to L4 molt, the male tail undergoes extensive reshaping and morphological changes. The tip of the tail retracts, cells in the tail region move anterodorsally, and connections between the alimentary tract, the gonad and the spicules are made. A pair of elongated spicules is formed with a layer of sclerotized refractile spicule cuticle laid down outside of each spicule and spicule retractor and protractor muscles connect to them. Another structure covered with the sclerotized refractile cuticle, called the

gubernaculum, lies posterior-dorsally to the spicules and also elongates and connects to sex muscles (Sulston et al., 1980). This structure presumably serves to guide the tips of the spicules ventrally through cloaca. Studies by Sulston et al. (1980) have suggested that the connection between male sex muscles and the proctodeal cells is essential for the anterodorsal elongation of the proctodeum during male tail reshaping at the late L4 stage. Ablation of muscle precursor cells results in a compressed proctodeum containing crumpled spicules and gubernaculum (Sulston et al., 1980). Electron micrographs reveal that the proctodeal cells B.a(l/r)appv form specialized contacts with the dorsal spicule retractors and protractors(Sulston et al., 1980). If these contacts are significant, the ε progeny B.a(l/r)appv may contribute to proctodeum reshaping.

To study spicule morphogenesis, we first dissected the contributions of each type of spicule cell to spicule morphogenesis and found that the spicule neurons and sheath cells are dispensable for spicule elongation but the spicule socket cells are essential for spicule morphogenesis. Ablation of all four socket cells in each spicule completely removes the spicule structure. The socket cells are required for two aspects of spicule morphogenesis: spicule elongation and secretion of spicule cuticle. The four socket cells of each spicule are partially redundant for these two functions. Seeking for socket cell marker, We found that *egl-17::GFP* is expressed in the spicule socket cells specifically during spicule morphogenesis and its expression pattern appears to be regulated between the socket cells. Using this marker we analyzed three types of mutants. Our results show that the socket cells are not only necessary but also sufficient to secret spicule cuticle. Spicules specifically

missing the spicule cuticle but otherwise wild-type can be formed. Thus the two functions of spicule socket cells are genetically separable. Furthermore, failure of socket cell movement results in crumpled spicules in mutants defective in TGF-β signaling. Thus the socket cell movement during spicule morphogenesis requires the TGF-β signaling pathway.

MATERIAL AND METHODS

Strains and Alleles

Routine culturing, maintenance and genetic manipulations of *C. elegans* strains were performed according to standard procedures (Brenner, 1974). N2 (Bristol) strain was used as wild type (Brenner, 1974). The following strains were used and are referenced in "*C. elegans* II" or as indicated below.

LG I: *ayIs4[egl-17::GFP+dpy-20(+)]* (Burdine et al., 1998)

LG II: *sma-6(e1482)*

LG III: *lin-48(sy548)* (this study), *sma-2(e502), sma-3(e491), sma-4(e729), dpy-17(e164)*

LG IV: *dpy-20(e1282), dpy-20(e1362)*

LG V: *him-5(e1490), syIs20[gpa-1::lacZ+dpy-20(+)]* (J. Mendel, and P. W. S., unpublished)

LG X: *sy170* (K. Liu, Y. Hajdu-Cronin, H. M. Chamberlin, and P. W. S., unpublished)

Anatomy and Cell ablation

Animal anatomy and cell division patterns were examined under Nomarski optics at 20°C as described by Sulston and Horvitz (1977). For laser ablation, L3 males were mounted on 5% agar pad and anesthetized with 1-3% sodium azide. Laser killing cells was performed as described (Sulston and White, 1980; Avery and Horvitz, 1987, 1989). Worms were then recovered from the agar pad and allowed to grow to adulthood.

GFP expression and Photographs

GFP expression was examined using a GFP filter set. Photographs were taken with Fuji Provia ASA 400 film.

RESULTS

Socket cells are essential for spicule morphogenesis

Each *C. elegans* male has a pair of spicules (Figure 2A). Each spicule is composed of two neurons, two sheath cells, and four socket cells. Previous studies have shown that each spicule neuron has a specific function during male mating behavior (Liu and Sternberg, 1995). However, we found that elimination of all spicule neurons and spicule sheath cells by cell ablation does not affect spicule morphology (Figure 2B). Since the male sex muscles have to attach to spicules to mediate spicule protraction and retraction during male mating behavior, and the socket cells form the outer layer of the spicules, we hypothesized that the socket cells might be essential for spicule

morphology. We tested this hypothesis by a series of cell ablation experiments.

We ablated the four socket cells on one side at the end of L3 lethargus while leaving the other side intact. When the males grew to adulthood, we found that only one elongated spicule is formed on the intact side (left) and no spicule structure or spicule cuticle material is formed on the socket cell-ablated side (right) (n=4, Figure 2C). Therefore, the socket cells are essential for spicule morphology including both the secretion of spicule cuticle and spicule elongation. We noticed that on the socket cell-ablated side the proctodeal cells are apparently posterior to their normal positions. Elimination of the socket cells thus also affects the overall proctodeum elongation to some extent. Sulston et al. (1980) suggested that the contact between the B.a(l/r)appv cells and sex muscles might be important for spicule elongation. When we ablated the B.a(l/r)ap cells in the early L3 stage, we found that the spicule structure formed might be slightly shorter than wild type but is otherwise wild-type (n=5). Ablation of the δ cells does not affect the spicule morphology (n=5).

Each of the four socket cells is derived from one of four different sublineages: α, β, γ, and ζ. Elimination of a single precursor at the late 10-cell stage does not affect the overall spicule morphology. We conclude that the four socket cells have redundant functions. To further address this question, we ablated two or three socket cells for each spicule. Elimination of two or more socket cells results in short and crumpled spicules. However, as long as a single socket cell is present, the sclerotized refractile cuticle material can be

generated. Therefore, the four socket cells of each spicule appear to have overlapping and partially redundant functions for spicule elongation and secretion of spicule cuticle. In intact animals, all socket cells fuse together and thus all contribute to spicule morphology.

The gubernaculum structure, which is posterior to the spicules and is thought to play a guidance role for spicule movement during mating behavior, is also covered by a layer of sclerotized cuticle. Transverse section of the male tail region revealed by electron micrographs suggests that the B.paa cell may be responsible for secreting the gubernaculum cuticle (Sulston et al., 1980). We confirmed this by cell ablation experiment. Ablation of B.paa cell is able to remove the gubernaculum structure while the spicules remain intact (n=5, Figure 2D).

egl-17::GFP is expressed in socket cells

During the mid-L4 to L4 lethargus stage, the male tail undergoes extensive reshaping and cells in the male tail region move extensively and change their morphologies. It is difficult to follow the cell movement and to judge cell fate based on their relative nuclear positions and morphologies of spicule cells. We therefore sought for cell fate specific markers expressed in the male tail region. We have found that *egl-17::GFP* is a marker for the spicule socket cells. *egl-17* encodes a fibroblast growth factor (FGF) and plays an important role for sex myoblasts migration in both hermaphrodites and males (Stern and Horvitz, 1991; Burdine et al., 1997). In males, we found that *egl-17::GFP* expression is turned on in several cells of the B lineage and the Y.p progeny Y.p(l/r)aa during L3 lethargus. The cells from the B lineage

appear to be two of the socket cells one on each side (likely α(l/r)d), B.paa, and B.pppa (Figure 3A). These *egl-17::GFP* expressing cells move anterodorsally during male tail reshaping. The movement of the socket cells is especially dramatic and the travel distance is about 20-25 μm. At the end of L4 lethargus, *egl-17::GFP* expression can be detected in a cluster of socket cells per spicule (Figure 3B). At this stage the socket cells have begun to fuse with each other. Shortly after the male reaches adulthood, *egl-17::GFP* expression only remains on in B.paa cell (Figure 3C).

To confirm this expression pattern, we performed cell ablation experiments. Ablation of the B.pa or B.paa cell eliminates the expression in the B.paa cell (n=5). When β and ζ cells are ablated in the early L3 stage, which eliminates the inner socket cells, the expression pattern is similar to that of intact animal (n=10; Figure 3D). Interestingly, when α and γ cells are ablated which eliminates the outer socket cells, we found that *egl-17::GFP* is still expressed in two cells in the spicule region (n=10; Figure 3E). The position of the cells appears to be ventral to that in intact animals and those cells are likely to be the socket cells derived from the β lineage. If α, γ, and β cells are ablated in the early L3 stage, still two cells in the spicule region are able to express the *egl-17::GFP* marker although the expression is weak (n=8). Based on the cell positions and nuclear morphologies, we think those cells are the socket cells derived from the ζ lineages. Only when all the socket cells are ablated is the expression eliminated (n=6). Thus, the socket cells seem to be able to communicate with each other to regulate *egl-17::GFP* expression. *egl-17::GFP* appears to be usually expressed in the most anterior

socket cell while inhibited in the more posterior socket cells. When the most anterior socket cell is eliminated, its expression can be turned on in the next most anterior socket cell. The regulation of *egl-17::GFP* expression is consistent with our observation that the socket cells have partially redundant function.

Ectopic socket cells produce ectopic spicule cuticle material in lin-48 mutants

In a genetic screen for males with abnormal spicules (Chapter III), we recovered a mutant with an ectopic spicule phenotype. This mutation, *sy548*, is an allele of *lin-48*, (Chamberlin, 1994; H. M. Chamberlin, P. W. Sternberg, and J. H. Thomas, in preparation). In *lin-48(sy548)* mutant males, a pair of crumpled spicules are formed at the normal position. In addition, an ectopic spicule-like structure is formed anterior-ventral to the crumpled spicules (Figure 4A). To determine the composition of this ectopic spicule structure, we examined the expression patterns of markers for two different cell types. We first examined the expression pattern of the socket marker *egl-17::GFP* in *lin-48(sy548)* mutant animals. Ectopic marker expressing cells are observed in the pre-anal region (Figure 4B). The U lineage is abnormal in *lin-48* mutants (Chamberlin, 1994; H. M. Chamberlin, P. W. Sternberg, and J. H. Thomas, in preparation). We therefore ablated the left/right pair of Ul/r cells in the early L3 stage and found this ablation eliminates the ectopic marker expressing cells and the ectopically formed spicule structure (n=5). We also examined the expression pattern of a spicule neuron specific marker, *gpa-1::lacZ* (J. Mendel and P. W. S., unpublished; Chapter III). The *gpa-1::lacZ*

marker is normally expressed in the spicule SPD neurons and phasmid neurons in the tail region. In *lin-48* mutant males, the marker expression is never observed in the ectopic spicule region (Figure 4A, n=20). The *lin-48* mutant also has defect in B cell progeny. In ~50% of the animals the *gpa-1::lacZ* marker fails to be expressed in SPD of the normally positioned crumpled spicules (n=50). These results suggest that ectopic spicule socket cells are sufficient to produce spicule cuticle and result in a spicule-like structure.

sy170 mutant males lack spicule cuticle but have elongated wild-type spicules

sy170 was originally recovered from a screen for copulation-defective mutants (K. Liu, Y. Hajdu-Cronin, H. M. Chamberlin, and P. W. S., unpublished). This mutant has normally elongated spicules but the sclerotized refractile cuticle material appears to be missing (Figure 4C). The mutant spicules are transparent and flaccid. Mutant males can not mate. The gubernaculum structure also lacks the sclerotized cuticle in *sy170* mutant males. But *sy170* mutant animals do have wild-type larval and adult cuticle. All lineage analysis does not reveal any cell division abnormality. We examined both neuronal marker and socket marker expression in this mutant. The neuronal marker, *gpa-1::lacZ*, is detected in both the cell bodies and the neuronal dendrites through the spicules, indicating that the spicule neurons are fully differentiated (Figure 4C). Thus the lack of spicule cuticle in *sy170* mutants is not due to the cells lacking adulthood characteristics. Expression of the socket marker, *egl-17::GFP*, also appears to be normal in

sy170 mutant males (Figure 4D). The presence of socket cells would explain the wild type-shaped spicule structure. Taken together, we conclude that the *sy170* mutant is specifically defective in making spicule cuticle. As socket cells are responsible for secretion of spicule cuticle, and *sy170* mutant males do have socket cells and elongated spicules, the socket cell function for secretion spicule cuticle is uncoupled from socket cell fate specification as well as from its function for mediating spicule elongation.

Socket cells fail to move anterodorsally in mutants defective in TGF-β signaling

The *sma* class mutants were isolated based on their small body size (Brenner, 1974). This class of mutants also have defects in patterning the rays of the male tail (Baird et al., 1991). Molecular and genetic studies have shown that several *sma* mutants define components of the TGF-β signaling pathway. *sma-2*, *sma-3*, and *sma-4* encode SMAD family proteins (Savage et al., 1996). Besides the small body size and ray defects, these *sma* mutants also share a crumpled spicule phenotype. We wondered if the crumpled spicule phenotype is a result of failure of socket cell movement during male tail reshaping.

Using the *egl-17::GFP* marker we examined socket cell positions in several *sma* mutants. As a control, we first examined marker expression in *dpy-17(e164)* mutant males. *dpy-17* mutants have a small body size that is comparable to the *sma* mutants. However, in contrast to the *sma* mutants, *dpy-17* mutant males have wild-type spicules. As shown in Figure 5A-5D,

socket cells in *dpy-17* mutant males travel anterodorsally through a substantial distance during male tail reshaping at the end of L4 stage ($\Delta \cong$20-25μm, comparable to wild type). In *sma-3* and *sma-4* mutants, the socket cells fail to move anterodorsally even though they still express the *egl-17::GFP* marker. At the end of L4 stage, the socket cells still remain at the same positions as at early L4 stage (Δ<5μm). *sma-2* and *sma-6* mutants display a similar defect. We believe that the failure of socket cell movement is the cause of the crumpled spicule phenotype observed in the *sma* mutants. As the *sma* genes represent components of a TGF-β signaling pathway, we speculate that the TGF-β signaling pathway is involved in spicule elongation during spicule morphogenesis.

DISCUSSION

The socket cells are essential for secretion of spicule cuticle and spicule elongation during spicule morphogenesis

The male tail reshaping at the L4 stage is one of the most complex morphogenic events that occurs during *C. elegans* development. Similar to morphogenesis and organogenesis in other organisms, extensive cellular behaviors occur, including cell movement, cell contact, cell fusion, and cell reshaping (Sulston et al., 1980; Baird et al., 1991). Using cell ablation techniques, molecular markers specific for cell fates, and genetic mutants, we have begun to elucidate the process of spicule morphogenesis in *C. elegans* males. We found that the socket cells are essential for two aspects of spicule

morphogenesis: secretion of spicule cuticle and spicule elongation. The four socket cells of each spicule are partially redundant for these functions. In contrast, the spicule neurons and sheath cells are dispensable for spicule morphogenesis. Similar results have been obtained for the generation of other sensory structures. In *Drosophila* bristle development, the shaft cell is both necessary and sufficient to generate the bristle structure. Loss of shaft cells result in loss of bristles phenotype, while excessive shaft cells at the expense of neurons are able to generate more bristles (reviewed in Jan and Jan, 1994). In *C. elegans* male sensory ray development, elimination of ray structural cell eliminates corresponding ray structure. Therefore, generation of a sensory structure requires the structural cells rather than the neurons.

For the secretion of spicule cuticle, the socket cells are both necessary and sufficient. We think the socket cells act cell-autonomously to produce spicule cuticle. First, ectopic socket cells result in ectopic deposition of spicule cuticle material in *lin-48* mutants. Second, only ablation of all the spicule socket cells is able to eliminate the spicule cuticle. Ablation of combinations of other cells can not eliminate the sclerotized refractile cuticle material.

Moreover, the existence of the *sy170* mutant suggests that secretion of spicule cuticle is genetically separable from spicule elongation. Elongated spicules that lack detectable spicule cuticle are formed in *sy170* mutant males. The *sy170* mutant may define a component for spicule cuticle synthesis that is regulated by socket fate specific genes.

The dynamic expression pattern of egl-17::GFP might implicate a role of the egl-17/FGF signaling pathway in spicule morphogenesis

Spicule elongation requires coordinated behavior of several cell types. The sex-specific muscles, spicule socket cells and the proctodeal cells all contribute to spicule elongation and proctodeum elongation during male tail reshaping. Communication between the sex muscles and the spicule socket cells is important to mediate the connection of these two cell types which leads to spicule elongation.

We observed an dynamic expression pattern of *egl-17::GFP* during male spicule morphogenesis. This dynamic expression pattern might suggest that the *egl-17*-mediated FGF signaling pathway play a role in mediating sex muscle connection to the spicules. First, *egl-17::GFP* is expressed at the right time during male tail reshaping. Its expression in the male tail region is turned on right before male tail reshaping and turned off shortly after males reaching adulthood. Second, *egl-17::GFP* is expressed in the right cells to mediate sex muscle connection to the proctodeum. The socket cells and B.paa cell are essential for the morphogenesis of the spicules and the gubernaculum, respectively. *egl-17::GFP* is expressed in both cell types. As the contact between male sex muscles and socket cells is important for proctodeum morphogenesis, it is possible that the socket cells use the FGF signal EGL-17 to attract male sex muscle positioning and attachment to the proctodeum. However, *egl-17* mutant males have wild-type spicules and can mate well. Therefore redundant signaling pathways must be used to control male tail morphogenesis.

In hermaphrodites, *egl-17::GFP* is found to be expressed in vulval cells and this expression pattern has suggested a role of the *egl-17*-mediated FGF signaling pathway in mediating the precise positioning of sex myoblasts and

coordinating sex muscle attachment to vulval cells in the development of a functional egg-laying system (Burdine et al., 1997; 1998). The expression pattern of *egl-17::GFP* in males suggests a similar function of the FGF signaling pathway and supports this hypothesis.

The TGF-β pathway is involved in spicule elongation

Mutants that disrupt the TGF-β signaling display a crumpled spicule phenotype. Examination of *egl-17::GFP* expression pattern revealed that the spicule socket cells fail to move anterodorsally during male tail reshaping in those mutants. Therefore, the TGF-β signaling pathway may mediate the socket cell movement during spicule elongation. Both the sex muscles and the spicule socket cells are required for spicule elongation. However, we do not know whether the connection between the sex muscles and the socket cells occurs before or after the anterodorsal movement of the socket cells. Analyses of the expression patterns of the TGF-β pathway components and mosaic analyses would be necessary to address the site of action of the TGF-β pathway and how it functions in spicule morphogenesis.

Developmental pathway for spicule morphogenesis

We propose the following developmental pathway for spicule morphogenesis in *C. elegans* males (Figure 6). Specification of socket cell fate turns on socket cell-specific genes. The socket cell-specific genes activate two morphogenetic pathways. One pathway regulates the secretion of spicule cuticle. The gene defined by the *sy170* locus is involved in this pathway. The

other pathway controls the anterodorsal movement of the socket cells and the connection between sex muscles and the socket cells which leads to spicule elongation. The EGL-17/FGF and SMA/TGF-β signaling pathways may be involved in this process. The same developmental pathway applies to the formation of the gubernaculum structure.

ACKNOWLEDGEMENTS

We thank Jane Mendel for the *syIs20* strain, Michael Stern for the *ayIs4* strain, Katherine Liu and Yvonne Hajdu-Cronin for the *sy170* allele, Helen Chamberlin for discussion on *lin-48* mutants prior to publication and critical comments on the manuscript, members of the Sternberg lab for discussion and comments on the manuscript. This work was supported by the Howard Hughes Medical Institute, with which P. W. S. is an investigator.

REFERENCES

Avery, L., and Horvitz, H. R. (1987). A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. *Cell* **51**: 1071-1078.

Avery, L., and Horvitz, H. R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous-system of *C. elegans*. *Neuron* **3**: 473-485.

Baird, S. E., Fitch, D. H. A., Kassem, I. A. A., and Emmons, S. W. (1991). Pattern-formation in the nematode epidermis - determination of the arrangement of peripheral sense-organs in the *C. elegans* male tail. *Development* **113**: 515-526.

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

Burdine, R. D., Chen, E. B., Kwok, S. F., and Stern, M. J. (1997). *egl-17* encodes an invertebrate fibroblast growth-factor family member required specifically for sex myoblast migration in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA.* **94**: 2433-2437.

Burdine, R. D., Branda, C. S., and Stern, M. J. (1998). *egl-17(fgf)* expression coordinates the attraction of the migrating sex myoblasts with vulval induction *C. elegans*. *Development* **125**: 1083-1093.

Chamberlin, H. M. (1994). Cell fate specification during *Caenorhabditis elegans* male tail development. Ph. D. thesis. California Institute of Technology.

Jan, Y. N., and Jan, L. Y. (1994). Genetic control of cell fate specification in *Drosophila* peripheral nervous system. *Annu. Rev. Genet.* **28**: 373-393.

Liu, K. S., and Sternberg, P. W. (1995). Sensory regulation of male mating behavior in *Caenorhabditis elegans. Neuron* **14**: 79-89.

Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J. R. (1997). *C. elegans* II. (eds). Cold Spring Harbor Laboratory Press.

Savage, C., Das, P., Fimelli, A. L., Townsend, S. R., Sun, C. Y., Baird, S. E., and Padgett, R. W. (1996). *Caenorhabditis elegans* genes *sma-2, sma-3,* and *sma-4* define a conserved family of transforming growth factor β pathway components. *Proc. Natl. Acad. Sci. USA.* **93**: 790-794.

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

Sulston, J. E., Albertson, D. G., and Thomson, J. N. (1980). The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. *Dev. Biol.* **78**: 542-576.

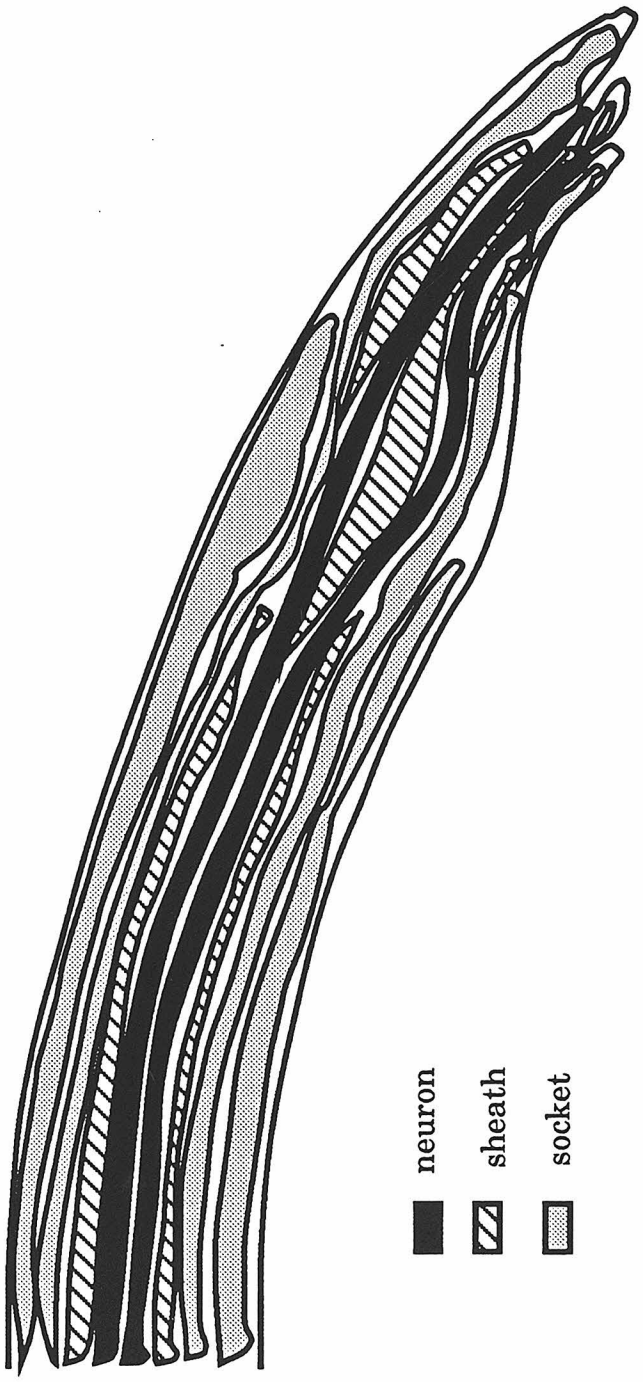
Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**: 577-597.

FIGURES

Figure 1. The composition of *C. elegans* male spicule and the spicule lineage.

(A). Diagram of longitudinal section of a male spicule (adapted from Sulston et al., 1980). Each spicule is composed of two neurons, two sheath cells, and four socket cells. Only cell processes are shown in this view. The sheath cells and the socket cells have begun to fuse. Solid line indicates neurons, slashed area indicates sheath cells, gray area indicates socket cells.

(B). The male B lineage (Sulston et al., 1977; Chamberlin and Sternberg, 1993). The B cell generates 10 progeny at the end of the L2 stage. The 10 cells continue to divide to generate spicule neurons, sheath cells, socket cells, and proctodeal cells. The spicule neurons and sheath cells are derived from the β and ζ lineages, the socket cells are generated by α, β, γ, and ζ lineages. sh denotes sheath cell, so denotes socket cell.



(A).

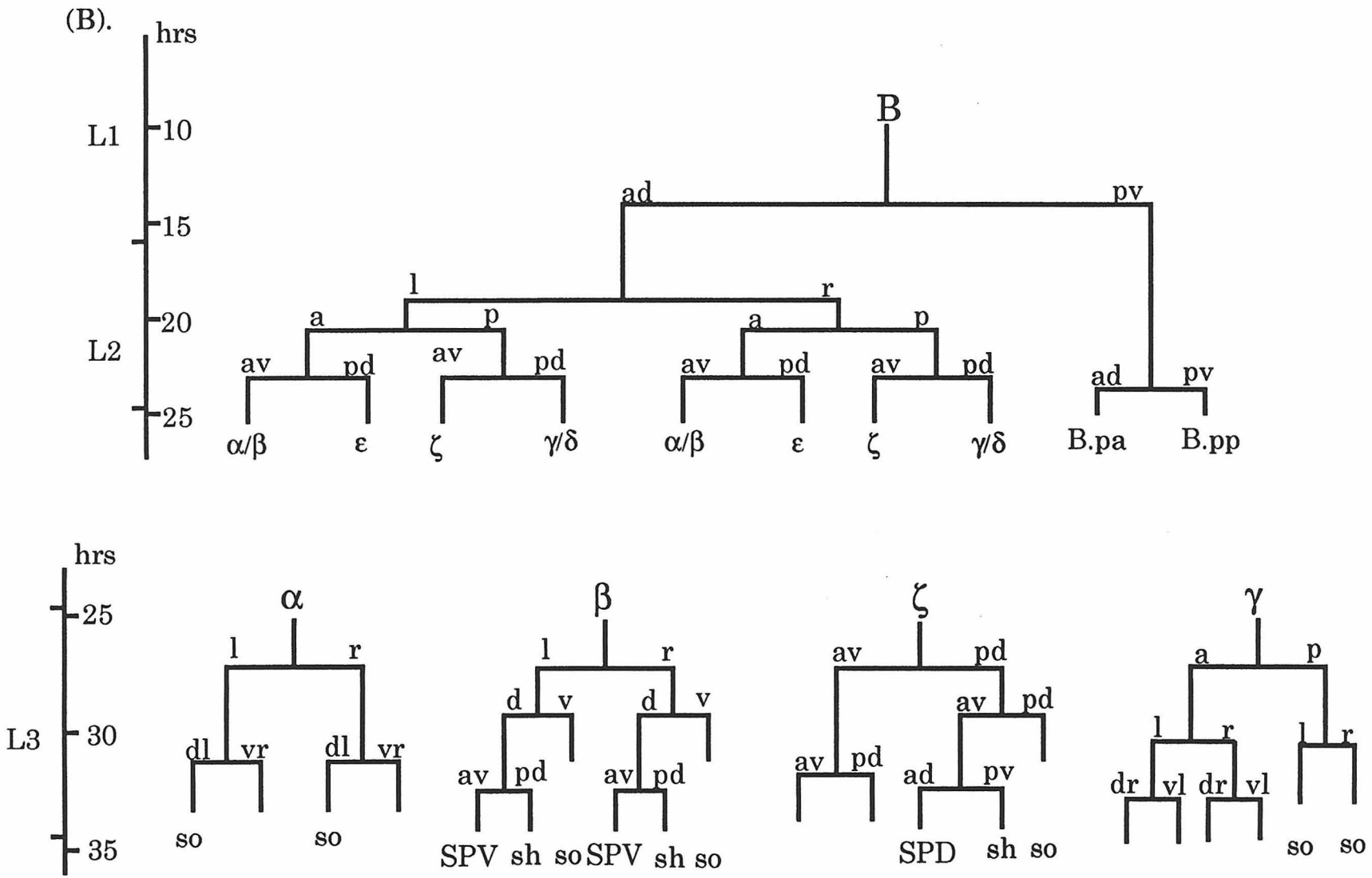

(B).
hrs
L1
10
15
L2
20
25
B
ad
pv
l
r
a
p
av
pd
α/β
ε
ζ
γ/δ
B.pa
B.pp
L3
30
35
α
β
ζ
γ
dl
vr
d
v
so
SPV
sh
SPD
dr
vl

Figure 2. The socket cells and the B.paa cell are responsible for spicule morphology and gubernaculum morphology, respectively.

(A). Wild type male tail, dorsal view.

(B). Elimination of all spicule neurons and sheath cells does not affect spicule morphology. All the spicule neurons and sheath cells were ablated in this animal, lateral view showing the left spicule.

(C). Ablation of socket cells eliminates spicule structure. All four socket cells on the right side were ablated at early L4 stage, and the right spicule structure is not formed. The left side remained intact, and a wild-type spicule structure is formed (sub-dorsal view).

(D). Ablation of the B.paa cell eliminates the gubernaculum while the spicules remain intact. (dorsal view, the left spicule is intact and is out of focus).

Arrow indicates spicules, arrowhead indicates the gubernaculum structure.

Scale bar indicates 20μm.

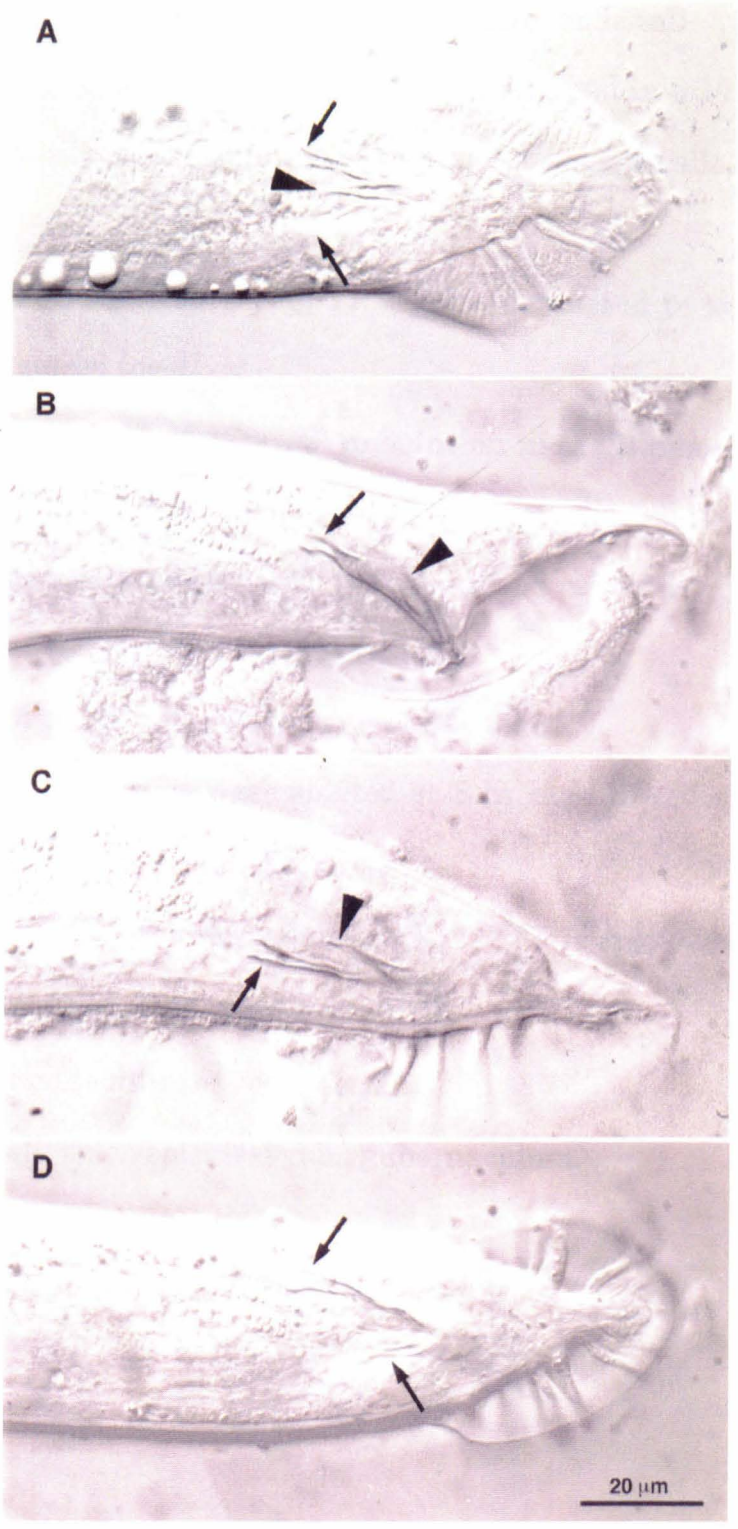

A
B
C
D
20 μm

Figure 3. *egl-17::GFP* expression pattern in the male tail.

(A). At the early L4 stage, *egl-17::GFP* is expressed in α(l/r)d cells, Y.p(l/r)aa cells, B.paa cell, and B.pppa cell. so denotes socket cells, here it points to α(l/r)d cell.

(B). At the end of L4 stage, *egl-17::GFP* is expressed in several socket cells that are clustered together.

(C). In adult males, *egl-17::GFP* remains on in the B.paa cell. spic. and gub. denote spicules and gubernaculum, respectively. These structures show aotufluorescence associated with the sclerotized refractile cuticle.

(D-E). Socket cell position at early L4 stage.

(F-G). Socket cell position at the end of L4 stage.

(H). When β and ζ cells were ablated at B lineage 10-cell stage, *egl-17::GFP* expression is similar to intact animals.

(I). When α and γ cells were ablated, *egl-17::GFP* is expressed in the socket cells derived from β lineage, β(l/r)v.

(D-I). arrowhead indicates socket cell.

so.: socket cell; spic.: spicules; gub.: gubernaculum.

Scale bar indicates 20μm.

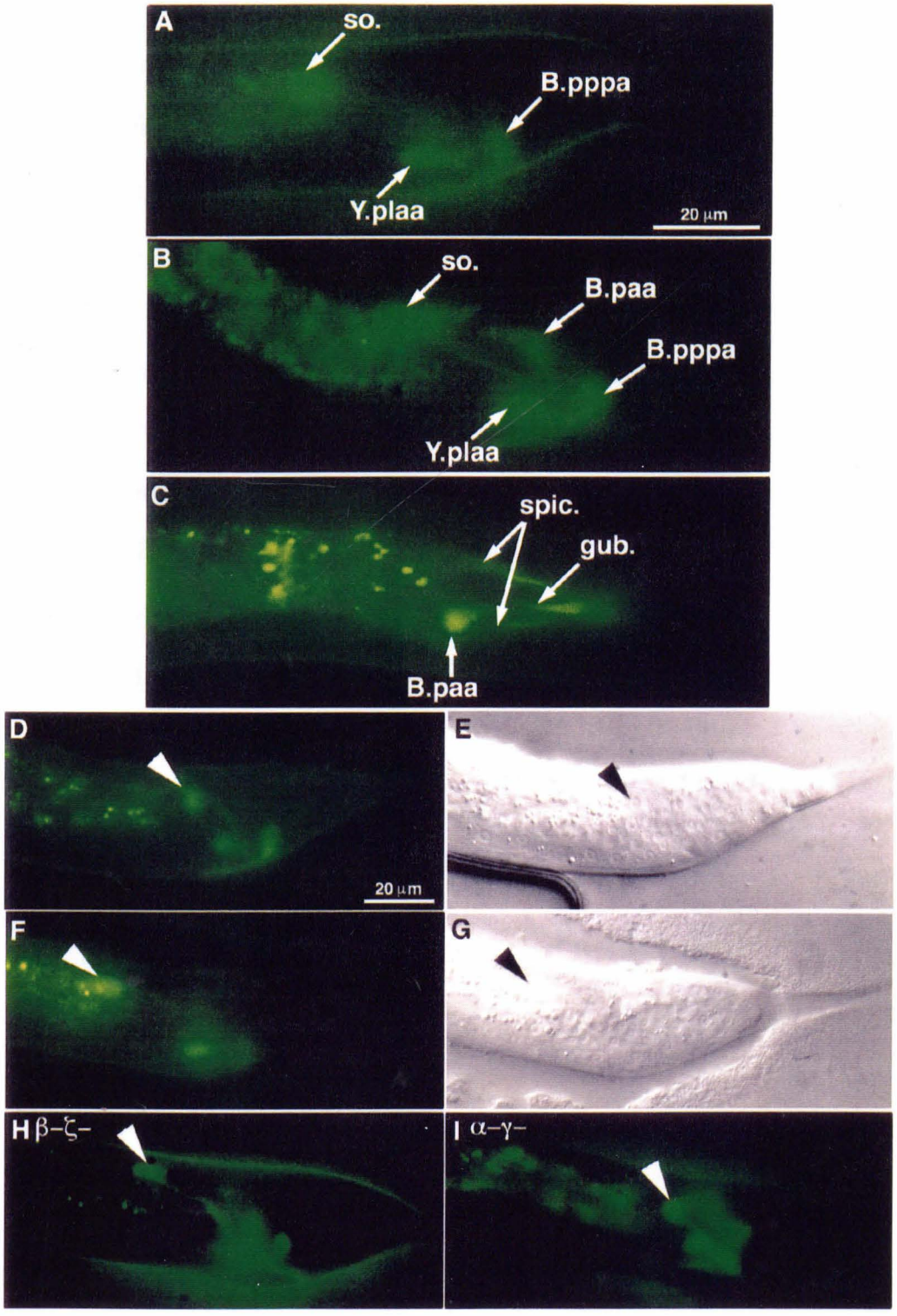

A
so.
B.pppa
Y.plaa
20 μm
B
so.
B.paa
B.pppa
Y.plaa
C
spic.
gub.
B.paa
D
20 μm
E
F
G
H β–ζ–
I α–γ–

Figure 4. Phenotypes and marker expression patterns in *lin-48(sy548)* and *sy170* mutants.

(A). *gpa-1::lacZ* expression in *lin-48(sy548)* mutant males. Arrow indicates ectopic spicule-like structure.

(B). *egl-17::GFP* expression in *lin-48(sy548)* mutant males. Arrow indicates ectopic socket cell, arrowhead indicates the normal socket cell.

(C). *gpa-1::lacZ* expression in *sy170* mutant. Adult males have transparent spicules that lack spicule cuticle. Marker expression is detected in both neuron cell bodies and dendrites through the spicules.

(D). *egl-17::GFP* expression in *sy170* mutant.

Arrowhead indicates socket cells.

Scale bar indicates 20μm.

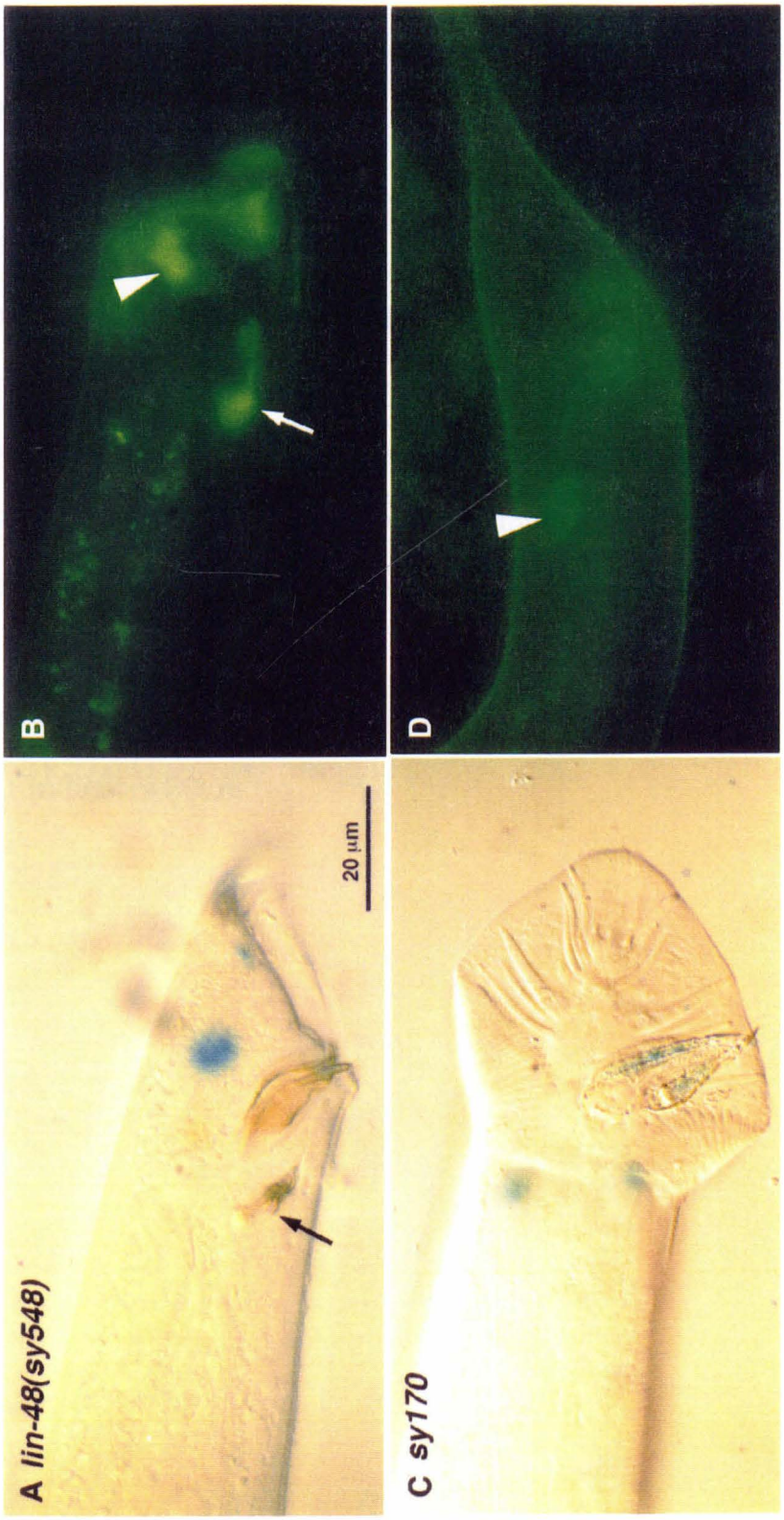

A lin-48(sy548)
20 µm
B
C sy170
D

Figure 5. *egl-17::GFP* expression in *sma* mutants.

(A-D). *dpy-17(e164)*

(E-H). *sma-3(e491)*

(I-L). *sma-4(e729)*

Left panel is GFP expression image, right panel is Nomarski image of the same animal. For each mutant, upper panel shows the expression pattern at early L4 stage, lower panel shows the expression pattern at late L4 stage. The travel distance of the socket cells is measured as the difference in the distances between the socket cells and the Y.plaa cell at the early and late L4 stages.

Arrowhead indicates socket cells.

Scale bar indicates 20μm.

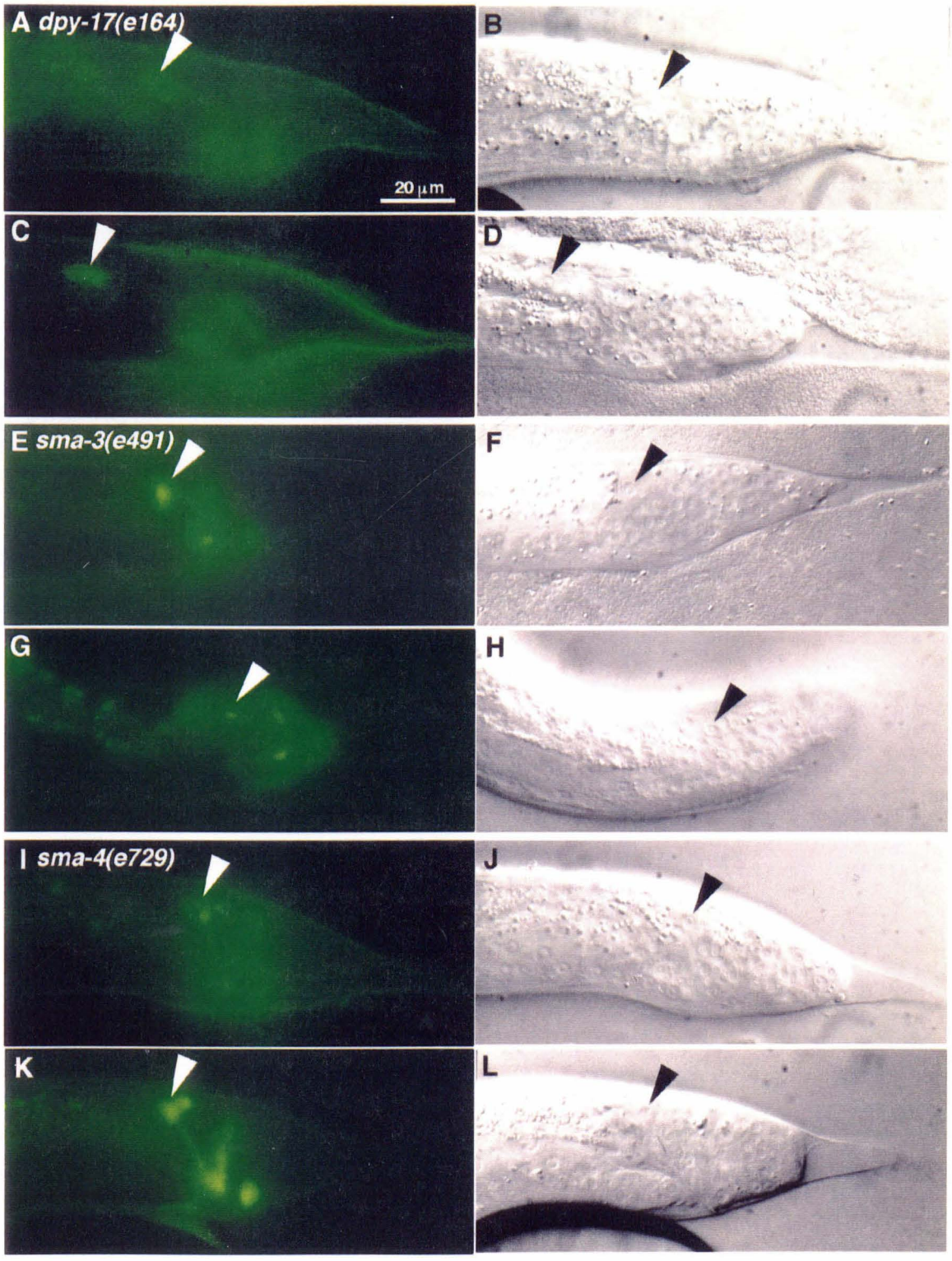
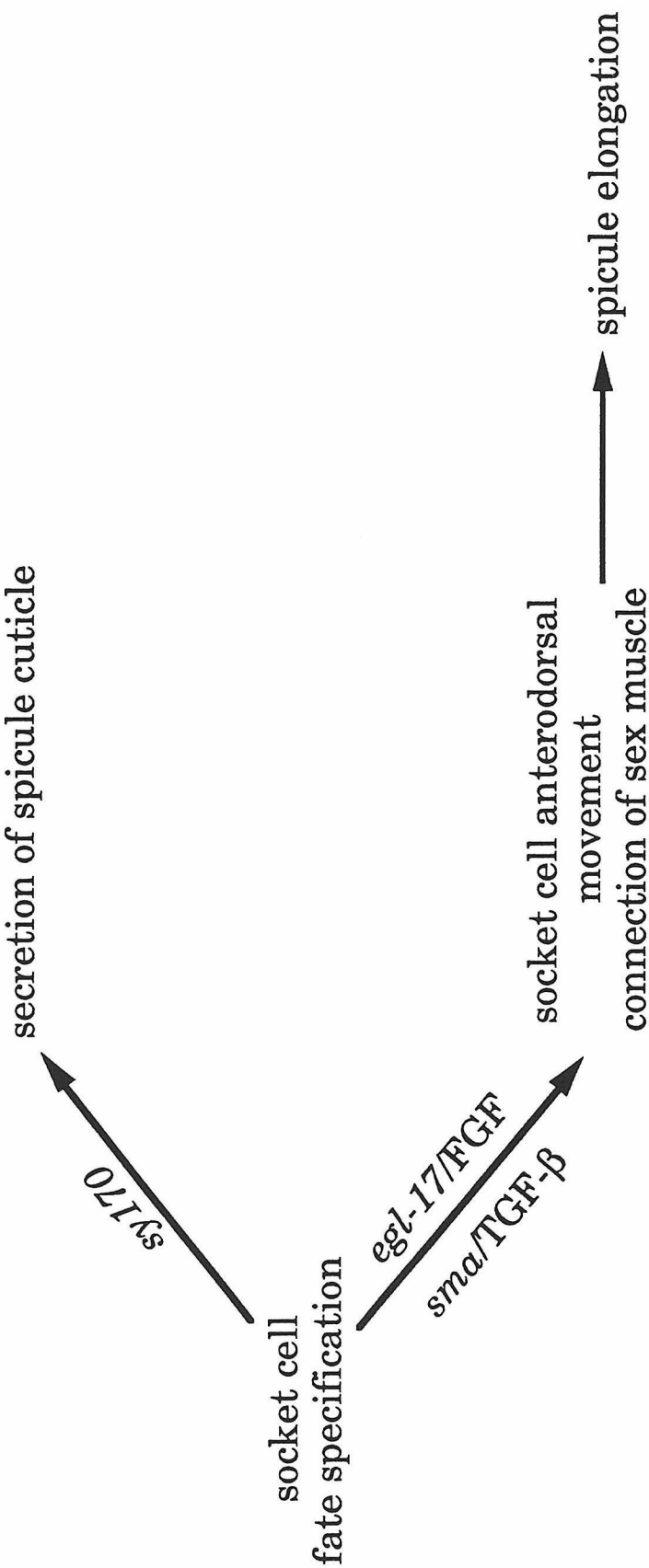

A dpy-17(e164)
20 μm
B
C
D
E sma-3(e491)
F
G
H
I sma-4(e729)
J
K
L

Figure 6. Developmental pathway of spicule morphogenesis.

Specification of the socket cell fate activates two parallel developmental pathways. One pathway controls the synthesis of spicule cuticle. The other pathway mediates spicule elongation by regulating the anterodorsal movement of the socket cells and the connection between the sex muscles and the socket cells. The gene defined by the *sy170* locus is involved in the former pathway. The FGF and TGF-β signaling pathways are involved in the latter pathway.


socket cell
fate specification
sy170
secretion of spicule cuticle
egl-17/FGF
sma/TGF-β
socket cell anterodorsal
movement
connection of sex muscle
to socket cells
spicule elongation

Chapter VI

SUMMARY

This thesis started with the attempt to unveil the strategies that nature has evolved for cell fate specification and regulation of cell functions. The development of an organism starts from a single totipotent cell. Cell proliferation, cell fate specification, regulation of cellular behavior and cell function, and generation of certain morphology and organs constitute the stepwise process of development. Each developmental decision is usually controlled by multiple extrinsic factors and intrinsic factors and the interactions between them. For the specification of a certain cell fate and cell function, there are three fundamental questions to be addressed. First, what genes are involved in the specification of a certain cell fate? Second, how do multiple extrinsic and intrinsic factors interact to make this developmental decision? Third, what genes control cellular behaviors and the exertion of cell functions? I have concentrated on the patterning of *C. elegans* tail in hope of gaining some insights on these developmental issues and to better understand the general principles of development. In this summary, I would like to compare *C. elegans* tail patterning with cell fate specification and patterning in other tissues and other organisms and to discuss several general principles and concepts of animal development.

During the last two decades several developmentally important signaling pathways have been identified, such as the EGF, Notch, Wnt, Hedgehog (Hh), and TGF-β signaling pathways (reviewed in Sternberg and Alberola-Ila, 1998; Greenwald, 1998; Cadigan and Nusse, 1997; Ingham, 1998; Whitman, 1998). Animal development, although complex, is mainly controlled by these several signaling pathways. The complexity of development is generated by the reiterative use of these major signaling

pathways in different tissues and at different developmental times. Yet each developmental decision often involves interactions of multiple pathways. For example, the RAS signaling pathway controls at least four different developmental processes during *C. elegans* development, viability, vulval induction, P12 fate specification, and male spicule development (Han and Sternberg, 1990; Aroian and Sternberg, 1991; Chamberlin and Sternberg, 1994; Chapter II). The Wnt signaling pathway is used to mediate asymmetric cell divisions from the first to the last cell division through B lineage development (Chamberlin and Sternberg, 1995; Chapter IV). While a simple decision of the P12 fate specification involves interactions of the RAS signaling pathway, the Wnt signaling pathway, and the hox genes (Chapter II). This seeming paradox raises several interesting questions.

First, if a certain signaling pathway is utilized to control several developmental processes, how is the specificity conveyed and how is the developmental diversity generated? Tissue specificity can be generated through the interactions between tissue specific genes and the signaling pathways. For example, activation of the RAS signaling pathway in the vulva precursor cells (VPC) induces vulva fate specification, while it promotes P12 fate specification in the tail region. The specificity is generated in part by the regional expression of the hox genes. The hox gene *lin-39*, a homologue of *Drosophila Sex combs reduced*, is expressed in the VPCs (Maloof and Kenyon, 1998). *lin-39* expression first generates a permissive condition for the VPCs to respond to the RAS signaling, later it is regulated by the RAS pathway to mediate the specification of certain vulval fates (Clandinin et al., 1997; Maloof and Kenyon, 1998; Eisenmann et al., 1998). For the specification of

the P12 fate, the hox gene *egl-5*, which is expressed in the tail region, acts as the downstream target of the RAS pathway to convey the tissue specificity of RAS signaling (Wang et al., 1993; Chapter II). Nuclear factors *lin-1* and *lin-31* may also contribute to the specificity of RAS signaling. *lin-1* appears to be a general downstream component of RAS signaling and is involved in several RAS mediated developmental processes, including vulva induction and the P12 fate specification (Tan et al., 1998). *lin-31* is specifically involved in vulva induction but not in the P12 fate specification and it is expressed in all the VPCs but not in the P12 cell (Tan et al., 1998). LIN-1 and LIN-31 form a protein complex to mediate vulva fate specification (Tan et al., 1998). It is possible that a LIN-31-like winged-helix transcription factor acts as a co-factor for LIN-1 in the P12 cell to convey the specificity of RAS signaling.

Second, why does a certain developmental decision usually involve many different pathways? Specification of cell fate and cell function is a stepwise process and requires coordinated physiological changes of cellular behaviors. In many cases, each aspect of cellular behavior is controlled by a different pathway (Chapter V). Involvement of multiple signaling pathways for a certain developmental decision may allow fine tuning of the decision. Communications between those signaling pathways can also coordinate several related developmental decisions. Depending on the interactions between those multiple pathways, inactivation of a single pathway may be partially compensated by other pathways or interfere with the functions of other pathways.

Third, what is the relation between the signaling pathways and their cellular functions? Although each signaling pathway controls several aspects

of cellular behaviors, there exists a general correlation between certain signaling pathways and certain cellular behaviors. For example, the EGF signaling pathway promotes cell proliferation and cell fate specification, the Notch/Delta signaling pathway mediates cell-cell interactions between developmentally equivalent cells, and the Wnt pathway controls asymmetric cell divisions and tissue polarity. This kind of correlation is determined in part by the nature of the signaling pathway. Diffusable signaling molecules usually can function across several cell diameters. A gradient of signaling molecules can be formed centering the signal source, thus making them ideal for generating a pattern of cell fate specification. The Notch/Delta signaling pathway requires cell-cell contact to transduce signaling activity. Relative level of the signal and receptor usually determines the activity of the pathway. Therefore, interaction between the cells of an equivalence group via the Notch/Delta signaling can make the cells different from each other. During male spicule development, the *lin-17*-mediated Wnt signaling pathway controls several asymmetric cell divisions through the B lineage development (Chamberlin and Sternberg, 1995; Chapter IV). One interesting feature of these asymmetric cell divisions is that the cytokinesis appears to be asymmetric resulting in two daughter cells with unequal size. The coupling of Wnt pathway and asymmetric cell division may reside in the regulation of cytokinesis and is reflected by the asymmetric expression level of the downstream transcription factor POP-1 (Lin et al., 1998). Due to the coupling between the signaling pathways and their functions, it has been possible to assess what kind of signaling pathway may be involved in certain developmental process by analyzing the nature of the process. The *C. elegans*

male spicule neurogenic lineage ζ, although resembling the *Drosophila* sensory organ precursor (SOP) lineage in its cell division pattern, is primarily regulated by the Wnt pathway rather than the Notch/Delta pathway (Chapter IV). The difference may be due to the apparently asymmetric cytokinesis that occurred during the development of the ζ lineage. The other spicule neurogenic lineage β has similar cell division pattern as ζ, yet is not regulated by the Wnt pathway. The cell divisions appear to be symmetric, making it resemble more the SOP lineage. This difference may suggest that the β lineage be regulated by the Notch/Delta pathway. Thus, the involvement of the Notch/Delta signaling pathway in *C. elegans* male spicule development will need to be further examined in the β lineage as well as in a sensitized *Wnt*⁻ background.

Fourth, is there any general coupling between signaling pathways for information integration? Although similar interactions between several signaling pathways have been observed in different developmental processes, there does not seem to exist tight linkage or general coupling of different signaling pathways for information integration. First of all, it has been demonstrated that two signaling pathways can act either antagonistically or cooperatively depending on the developmental context. Second, the spatial and temporal expression patterns of the signaling molecules determine if two signaling pathways would intersect for a certain developmental process. Changes of the expression patterns would affect how the two pathways interact. Third, two signaling pathways can intersect at multiple levels. Information integration at different levels may result in different outcomes.

This does not mean that there are no general rules for interaction of multiple pathways and information integration. Only variation of certain parameters can result in a variety of combinations and many different outcomes, and we have not explored or identified all the possibilities and mechanisms.

Fifth, how do we dissect the intersections of multiple signaling pathways? There are two directions to address this question. One direction is toward finding all possible interactions between multiple signaling pathways by qualitatively examining the interactions in various tissues and organisms. The other direction is to use model systems to quantitatively dissect the mechanisms of pathway interactions, such as at which level the signaling pathways intersect, how do they interact, and how does one pathway changes its activity when the activity of other pathways is changed. Toward this end, we need first to identify all the major components and regulators of those signaling pathways, and second to be able to monitor the activities of each component. A combined genetic, cellular, and biochemical approach has become more and more important for analyzing the functions of pathway regulators. In *C. elegans*, the *lin-2*, *lin-7*, *lin-10* mutants were identified from a genetic screen based on vulval induction phenotype. The functions of the genes defined by those three loci have not been revealed until recently (Kaech et al., 1998). The LIN-2, LIN-7, LIN-10 proteins interact with each other and with the LET-23 receptor tyrosine kinase and localize the LET-23 receptor to the proper cellular compartment for its function (Kaech et al., 1998). If it were not for the biochemical studies and in vivo protein localization assays, the role of those proteins would have remained a mystery. Chapter III of this thesis described a gene expression pattern-based genetic screen looking for

genes involved in the terminal cell fate specification during male spicule development. A weak allele of *lin-17* displays an abnormal marker expression pattern and confers a spicule sheath to neuron fate transformation (Chapter IV). This finding reveals the involvement of the Wnt signaling pathway in the neuronal vs. non-neuronal cell fate specification in *C. elegans* male spicule development. The genetic screen also identified a mutation in an HMG1/2-like gene that displays the same cell fate transformation phenotype. HMG1/2-like proteins are canonically known as chromatin structural proteins and are not expected to have specific developmental functions. Yet our genetic analyses suggest a specific role for the HMG1/2-like protein in Wnt signaling. One possibility may be that the *C. elegans* HMG1/2-like protein is different from the HMG1/2 proteins in other organisms and it may have unique properties and play specific roles during development. Alternatively, the identification of the involvement of an HMG1/2-like protein in Wnt signaling may suggest a link of the Wnt signaling to other cellular behaviors. Protein-DNA interaction, protein-protein interaction and other biochemical assays would be necessary to further dissect the function of this protein as well as the function of the Wnt pathway.

With the rapid progress in the field, biological sciences have come to an era in which we need to understand the fine tuning of developmental pathways and of signal transduction at protein level. The work of this thesis has identified the involvement of several signaling pathways in generating the tail patterning in *C. elegans*. The temporal and spatial interactions of those signaling pathways have begun to be elucidated. Further studies on the regulations and interactions of those signaling pathways will be much needed

to further understand the mechanisms of signaling transduction and pathway integration.

REFERENCES:

Aroian, R. V., and Sternberg, P. W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. Genetics 128: 251-277.

Cadigan, K. M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Gene & Dev. 11: 3286-3305.

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

Chamberlin, H. M., and Sternberg, P. W. (1995). Mutations in the *Caenorhabditis elegans* gene *vab-3* reveal distinct roles in fate specification and unequal cytokinesis in an asymmetric cell-division. Devel. Biol. 170: 679-689.

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

Eisenmann, D. M., Maloof, J. N., Simske, J. S., Kenyon, C., and Kim, S. K. (1998). The β-catenin homolog BAR-1 and LET-60 RAS coordinately regulate the hox gene *lin-39* during *Caenorhabditis elegans* vulval development. Development 125: 3667-3680.

Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. Gene. & Dev. 12: 1751-1762.

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

Ingham, P. W. (1998). Transducing Hedgehog—the story so far. EMBO J. 17: 3505-3511.

Kaech, S. M., Whitfield, C. W., and Kim, S. K. (1998). The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the *C. elegans* EGF receptor LET-23 in vulval epithelial cells. Cell 94: 761-771.

Maloof, J. N., and Kenyon, C. (1998). The hox gene *lin-39* is reuired during *C. elegans* vulval induction to select the outcome of Ras signaling. Development 125: 181-190.

Sternberg, P. W., and Alberola-Ila, J. (1998). Conspiracy theory: RAS and RAF do not act alone. Cell 95: 1-4.

Tan, P. B., Lackner, M. R., and Kim, S. K. (1998). MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. Cell 93: 569-580.

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

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. Gene. & Dev. 12: 2445-2462.