C. ELEGANS MALE TAIL DEVELOPMENT

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Hui Yu

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DEDICATION

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Abstract

The *C. elegans* hook sensillum is a male copulatory structure developed from the hook sensillum competence group (HCG), P(9-11).p. Male P(9-11).p each adopt one of three potential fates (1°, 2°, or 3°), forming an invariant spatial pattern of 3°-2°-1°. I examined HCG fate specification in various genetic backgrounds and found that both *lin-17* Frizzled-like Wnt receptor and *bar-1* β-catenin are predominantly expressed in 1° P11.p. Activation of the *bar-1* canonical Wnt pathway by a *pry-1*(Axin) mutation changes the competence of the anterior P(3-8).p cells and induces ectopic 1° HCG fate. I also found that the Hox gene *mab-5* acts downstream of the Wnt pathway to determine HCG competence. Ectopic *mab-5* expression, in conjunction with activated LIN-12 or EGF signaling, causes ectopic HCG fates in anterior P(1-8).p. Furthermore, epistatic interactions between *lin-17* and *lin-12* showed that generation of 2° HCG fate in P10.p by LIN-12/Notch activity depends on LIN-17-mediated Wnt signaling**.** Together these observations suggest that Wnt signaling is the major player that governs HCG patterning and functions in HCG competence, specification, and execution.

Precise execution of the 2° HCG lineage leads to generation of all five major components of a functional hook sensillum that mediates vulva location behavior during male mating. Both sensory neurons of the hook sensillum, HOA and HOB, are necessary for efficient vulva location. From a genetic screen for altered expression of an HOB marker *ceh-26::gfp* or abnormal hook morphology, I isolated five mutants. Further analysis of one of these mutants identified an allele of *egl-46*, a zinc-finger transcription factor. EGL-46, and to some extent, another transcription factor EGL-44, regulate a group of HOB-

specific genes. These targets include the homeodomain protein CEH-26, a neuropeptide-like protein NLP-8, a degenerin homologue T28F4.2, and two *C. elegans* polycystin homologs LOV-1 and PKD-2. Both *egl-46* and *egl-44* mutants exhibit defective vulva location behavior, suggesting impaired HOB function. The regulator of a general ciliogenic pathway, DAF-19, indirectly affects expression of HOB-specific genes. Expression of DAF-19, EGL-46, and EGL-44 is independent from one another, indicating that general and cell-specific regulatory factors act in parallel to produce cell specificities crucial for HOB sensory function.

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CHAPTER 1

Introduction

During the development of multicellular organisms, a common issue is how a cell becomes distinguished from its neighbors and acquires a unique identity. From nematodes to mammals, several major signal pathways have been shown to be involved in different developmental processes repeatedly to specify particular cell types, suggesting that instructions from common signaling pathways must be interpreted in a tissue-specific way to guide distinctive pattern formation. Two different but related questions need to be addressed: Is there a common scenario that directs individual fate specification, and if so, how is specificity generated. One example of multiple cell type determination is fate specification among cells of an equivalence group, in which cells share common developmental potentials but subsequently adopt distinct fates.

Comparison of three equivalence groups derived from *C. elegans* P lineages

Three different equivalence groups are generated by *C. elegans* postembryonic ventral P precursor cells (Sulston and Horvitz, 1977; Sulston and White, 1980). At hatching, 12 P cells are positioned in six bilateral pairs located symmetrically at each side along the body, which are called P1/2L, P1/2R, P3/4L, P3/4R, etc. At the middle L1 larval stage, P cells migrate ventrally into the ventral cord, and form a linear assembly along the anterior-posterior body axis, and are therefore renamed as P1-P12. The relative anterior-posterior order of the two P cells in a bilateral pair after the ventral migration is almost random for P3 to P10. For example, P3/4L can be either P3, if occupying the relative anterior position, or P4, if placed posteriorly. P1 is usually from the right side P1/2R. Only the P11/P12 pair has an invariant migration pattern: the left one becomes anterior P11 and the right one the posterior P12. P11 and P12 differ not only in their positions in

the ventral cord but also in the fates of their progeny (see below). However, if one cell of the P11/P12 pair is killed before migration, the remaining one always becomes P12, suggesting that the two cells have the same developmental potential to become P12 (Sulston and White, 1980). Therefore, the P11/P12 pair form an equivalence group with a binary fate choice (Sulston and White, 1980).

Shortly after entering the ventral cord, each Pn (n=1-12) cell divides once to generate a neuroblast cell (Pn.a), which produces neurons in the ventral cord, and a posterior daughter (Pn.p). In both sexes, the most posterior Pn.p cell, P12.p, generates two progeny in the late L1 stage. Its posterior daughter, P12.pp, goes through apoptosis before the L1 molt. The anterior daughter, P12.pa, is a hypodermal cell (hyp12) with a characteristic small nucleus that is distinct from the large nuclei of other Pn.p cells (Sulston and White, 1980). Therefore, a Pn.pa hyp12 cell is a marker of the P12 fate. Other Pn.p cells display sexually dimorphic developmental potentials. In hermaphrodites, the six central Pn.p cells, P(3-8).p, constitute the vulval precursor cell (VPC) equivalence group, whereas in males the three posterior Pn.p cells, P(9-11).p, comprise the hook sensillum competence group (HCG) (Sulston and White, 1980; Fig 1). The cells within each group have three different potential fates (1°, 2°, or 3°).

The hermaphrodite vulval equivalence group, P(3-8).p

Over two decades of research provides us with a comprehensive understanding of pattern formation during vulval development. Each of the six multipotent vulval precursors cells, P(3-8).p, is able to adopt one of three fates, 1°, 2°, or 3° (Sulston and White, 1980;

Kimble, 1981; Sternberg and Horvitz, 1986). The 1° and 2° fates have distinct cell division patterns and produce progeny that form the hermaphrodite vulva. The 3° fate is a non-vulval fate, in which the cell undergoes a single round of cell division and both progeny join the hyp7 epidermal syncytium. The wild-type spatial pattern of the VPCs, 3°-3°-2°-1°-2°-3°, is established by interactions between cells both inside and outside the vulval equivalence group (Sternberg and Horvitz, 1986). Multiple signaling pathways mediate these cell-cell interactions during different steps of vulval development (Greenwald et al., 1983; Ferguson et al., 1987; Sternberg and Horvitz, 1989; Moghal and Sternberg, 2003).

VPC competence

Cell competence refers to the potential of a cell to adopt particular cell fates. Establishment of VPC competence requires the activities of the Hox gene *lin-39* (Clark et al., 1993; Salser et al., 1993; Clandinin et al., 1997; Shemer and Podbilewicz, 2002). Five hermaphrodite Pn.p cells that do not express *lin-39*, P(1-2).p and P(9-11).p, fuse with hyp7 in the late L1 stage (Salser et al., 1993). Expression of *lin-39* in P(3-8).p allows these cells to escape fusion with the hyp7 syncytial epidermis and become vulval precursor cells (Clark et al., 1993; Salser et al., 1993; Shemer and Podbilewicz, 2002). A canonical Wnt pathway involving the APC–related gene *apr-1* (Hoier et al., 2000) and β-catenin *bar-1* (Eisenmann et al., 1998) regulates VPC competence by controlling *lin-39* expression. In ~50% of wild-type animals, P3.p does not divide, and undivided P3.p adopts the F (fused) fate in the late L2 stage (Sulston and White, 1980; Sternberg and Horvitz, 1986). Mutations in *bar-1* disrupt *lin-39* expression and cause other central Pn.p

cells, P(4-8).p, to similarly adopt the F fate in the late L2/early L3 stage (Eisenmann et al., 1998). Disruption of *apr-1* function in the Pn.p cells has a similar effect on *lin-39* expression and P(3-8).p fusion (Hoier et al., 2000). APR-1 probably positively regulates BAR-1 function in generation of the vulval equivalence group. A positive role for *apr-1* in Wnt signaling is also suggested by analysis of *C. elegans* embryonic development (Rocheleau et al., 1997). By contrast, in vertebrates and *Drosophila*, binding of APC to β-Catenin promotes degradation of β-Catenin proteins, and thus APC function is thought to be inhibitory for Wnt signaling (Reviewed by Wodarz and Nusse, 1998; Bienz, 1999). The *C. elegans* genome contains five Wnt genes: *lin-44*, *egl-20*, *mom-2*, *cwn-1*, and *cwn-2*, and four Fizzled-like Wnt receptors: *lin-17, mom-5, mig-1*, and *cfz-2* (Korswagen, 2002). The Wnt and the receptor required for VPC competence have not yet been identified.

VPC induction

Cell fate specification is the process by which a cell chooses its future developmental plan in response to specific cues. If the source of the cue resides outside the equivalence group, this type of fate specification is called induction. The anchor cell (AC) in the somatic gonad is located centrally dorsal to above the VPC equivalence group and produces an inductive signal to specify the 1° and the 2° VPC fates (Kimble, 1981; Sternberg and Horvitz, 1986). This inductive signal, LIN-3(EGF), is graded from the AC in both anterior and posterior directions. The nearest Pn.p cell, P6.p, receives a high level of LIN-3 signal from the AC and activates LET-23 (EGFR RTK)/LET-60(RAS)/MPK-1(MAPK) pathway to adopt the 1° fate, which then sends a lateral signal to activate LIN-

12(Notch) in the neighboring P5.p and P7.p. P5.p and P7.p assume the 2° fate in response to an intermediate level of LIN-3 signal and LIN-12 activation (Yochem et al., 1988; Katz et al., 1995; Koga and Ohshima, 1995; Simske and Kim, 1995). The more distant P(3, 4, 8).p are not induced due to lack of sufficient LIN-3 signal and adopt the 3° fate (Katz et al., 1995). Loss of EGF signaling by either AC ablation or genetic mutations in either *lin-3* or major components of *let-23/let-60* pathway causes all P(3-8).p to adopt the 3° fate, resulting in a vulvaless (Vul) phenotype (Sternberg and Horvitz, 1989; Aroian et al., 1990; Beitel et al., 1990; Han et al., 1990; Clark et al., 1992; Hill and Sternberg, 1992; Han et al., 1993; Lackner et al., 1994; Wu and Han, 1994; Kornfeld et al., 1995; Wu et al., 1995; Chang et al., 2000). In contrast, excessive EGF signaling renders additional 1° or 2° VPC induction in P(3, 4, 8).p cells, generating a multivulva (Muv) phenotype (Beitel et al., 1990; Han et al., 1990; Clark et al., 1994; Huang et al., 1994; Katz et al., 1996; Wang and Sternberg, 1999). The *lin-3* inductive signal most likely has two functions: to distinguish vulval fates from the non-vulval 3° fate, and to specify the 1° VPC fate (Sternberg and Horvitz, 1986; Katz et al., 1995).

Maintaining LIN-39 activity is not only required for VPC competence, but is also necessary for the normal response to LIN-3 inductive signals (Clandinin et al., 1997; Maloof and Kenyon, 1998). During vulval induction, *lin-39* expression is upregulated by activated RAS pathway and peaks in the presumptive 1° cell, P6.p (Maloof and Kenyon, 1998). Cell divisions in the 1° and 2° vulval lineages are blocked by loss of *lin-39* activity at the time of induction (Clandinin et al., 1997; Maloof and Kenyon, 1998). On the other hand, overexpression of *lin-39* does not cause vulval overinduction as Muv

mutants do, suggesting a permissive role of *lin-39* in VPC fate specification (Maloof and Kenyon, 1998). No requirement for Wnt signaling has been found during normal vulval induction. However, hyperactivation of a canonical Wnt pathway by either a *pry-1*(Axin) mutation or overexpression of a stabilized β-catenin protein delNT BAR-1 (an N-terminal truncation that removes GSK3β phosphorylation sites) from a heat shock promoter could induce extra vulval fates in P(3, 4, 8).p cells (Gleason et al., 2002).

The *lin-12* ligand(s) made by the 1° VPC did not emerge in the picture of vulval induction until recently. Out of ten genes identified in *C. elegans* that contain a Delta/Serrate/Lag-2 (DSL)-like motif and thus are candidate *lin-12* ligands, three (*apx-1*, *dsl-1*, and *lag-2*) have been shown to function redundantly as parts of the lateral signal (Chen and Greenwald, 2004). However, the combination of double feeding RNAi of *apx-1* and *lag-2* in *dsl-1(ok810)* deletion mutants to decrease activities of all three DSL proteins only causes a minor vulval defect in a non-sensitized genetic background (Chen and Greenwald, 2004), leaving a open question as to whether other components contribute to the lateral signal. In P5.p and P7.p, activation of LIN-12 signaling elevates the expression of several genes that negatively regulate the MAPK pathway, including a MAP kinase phosphatase gene, *lip-1* (Berset et al., 2001; Yoo et al., 2004). The initial activation of LET-23-MAPK pathway in P5.p and P7.p by the LIN-3 signal might trigger these two cells into induced vulval fates but subsequent downregulation of the MAPK pathway by LIN-12 signaling may restrict P5.p and P7.p to the 2° fate (Sternberg and Horvitz, 1986; Katz et al., 1995; Yoo et al., 2004).

VPC execution

Cell fate execution is all the events subsequent to specification. Here, fate execution can be assayed by the number of cell divisions and types of progeny produced. The 1° VPC fate execution requires continual activation of RAS signaling by the AC (Wang and Sternberg, 2000). Similarly, LIN-12 signaling is probably necessary for the 2° VPC fate execution (Yoo et al., 2004). In addition, *lin-17* Wnt receptor and *lin-18* (RTK) act in parallel to maintain the proper orientation in both the P6.p 1° lineage and P7.p 2° lineage (Wang and Sternberg, 2000; Inoue et al., 2004). *lin-17* and *lin-18* separately mediate signals from different Wnt ligands to control P7.p lineage orientation: LIN-44 probably interacts with LIN-17, MOM-2 interacts with LIN-18, and a third Wnt protein CWN-2 functions redundantly with LIN-44 and MOM-2 (Inoue et al., 2004).

The male hook sensillum competence group, P(9-11).p

The pattern of three fates in the male hook sensillum competence group is as follows: P9.p adopts the 3° fate, P10.p the 2° fate, and P11.p the 1° fate (Sulston and White, 1980; Herman, 1991). The similar cell origin of hermaphrodite VPCs and male HCG (different subsets of homologous Pn.p cells), similar three fate choices (Sulston and White, 1980), as well as the same requirement of LIN-12/Notch signaling for 2° fate specification (Greenwald et al., 1983), raises the possibility that *C. elegans* might utilize the same set of signaling pathways that function in vulval development for male HCG fate specification. However, it is not that case. The EGF pathway, the central signaling pathway inducing vulval development, is apparently dispensable in HCG patterning (P. W. Sternberg, unpublished). During VPC induction, EGF signal is graded from a signal

source, the gonadal anchor cell (AC). So far, no single cell or group of cells has been identified as the source of HCG inductive signal(s) (Sulston et al., 1980; Herman, 1991). The signal must be so diffusible that it is impossible to remove the signal by cell ablation. Alternatively, the signal source might be redundant and the correct combination of cells secreting the signals has not been discovered yet or is unable to be identified without killing the animal. The isolation of vulvaless (Vul) mutants that cause 1° and 2° VPC to become 3° greatly facilitated the uncovering of regulatory pathways involved in vulva development. Only three hookless mutants have been identified. (1) *mab-5* loss-of-function mutants have no HCG cells because of abnormal fusion of P(9-11).p with hyp7 in the L1 stage (Kenyon, 1986), and therefore are not informative about signals involved in HCG fate specification. (2) A *lin-12* loss-of-function mutation affects the P10.p 2° fate, resulting in the hookless phenotype (Greenwald et al., 1983). (3) A *lin-17* Wnt receptor mutant is also hookless due to abnormal cell divisions of P10.p and P11.p and aberrant differentiation of their progeny (Sternberg and Horvitz, 1988). However, the *lin-17* mutation usually does not block the P10.p and P11.p cell proliferation, making its role in HCG induction unclear.

The deficient hook formation in *lin-17* mutants provides us a starting point to explore function of Wnt pathway during HCG patterning. In the Chapter 2, I will present evidences that Wnt signaling plays an essential role during HCG patterning. First, it regulates *mab-5* activity for HCG competence. Second, it promotes the 1° fate in P11.p. Third, it functions at every cell division to ensure execution of 1° P11.p and 2° P10.p lineages. The Wnt signal(s) is probably graded from the posterior tail region. Multiple

cells in the male tail express different Wnt genes (Herman et al., 1995; Whangbo and Kenyon, 1999; work in the Chapter 2). The LIN-17 Frizzled-like receptor is expressed at a relatively higher level in the 1° P11.p lineage (Sawa et al., 1996; work in the Chapter 2). Downstream of LIN-17, β-catenin *bar-1* is preferentially expressed in P11.p and displays a dynamic pattern of subcellular protein localization at the time of the 1° fate specification (work in the Chapter 2). Activation of the canonical Wnt pathway by a mutation in *pry-1*(Axin) gene is sufficient to mis-express HCG competence in Pn.p cells anterior to P(9-11).p and promote ectopic 1° HCG fates (work in the Chapter 2). However, unlike LIN-17, the loss of BAR-1 function only causes a mild defect in hook formation (work in the Chapter 2). The substantial phenotypic difference between *lin-17* mutants and *bar-1* mutants implies that other branches of Wnt pathway participate in HCG pattern formation as well. It is also possible that different steps of HCG patterning (competence, specification, and execution) involve different Wnt-receptor interactions and different downstream pathways.

LIN-12 signaling mediates a lateral interaction between P11.p and P10.p (Greenwald et al., 1983). The 1° P11.p probably produces a LIN-12 ligand, which could activate LIN-12 signaling in the neighboring P10.p to promote the 2° fate in this cell. In the Chapter 2, I will present that expression of *lip-1* MAP kinase phosphatase, which has been shown to act downstream of LIN-12 during 2° VPC induction, is up-regulated in the presumptive 2° P10.p cell, but is down-regulated in the presumptive 1° P11.p cell during the late L2 stage, indicating that the activity of the MAPK pathway in these two cells may contribute to the 1°/2° fate decision. The MAPK pathway is thought to lie downstream of LIN-3

EGF signaling. So far, no obvious role in 1° HCG fate induction has been identified for EGF signaling. Activation of the LET-23 RTK pathway by *lin-15* mutations causes a 2°-like fate transformation in the normally tertiary P9.p cell (P. W. Sternberg, unpublished). A possible explanation is that the MAPK pathway might have opposing effects on the production of LIN-12 and its ligand(s), and reduction of MAPK activity not only ensures LIN-12 signaling in P10.p but also prevents generation of LIN-12 ligand(s) to inappropriately induce a 2° fate in adjacent P9.p.

The Hox gene *mab-5* not only prevents the fusion of P(9-11).p with hyp7 in the late L1 stage (Kenyon, 1986; Salser et al., 1993), but also provides these three cells with HCG competence (work in the Chapter 2). In the Chapter 2, I will show evidences that *mab-5* overexpression is sufficient to alter the cell competence in the anterior P(1-8).p cells. In a *mab-5* gain-of-function mutant, additional LIN-12 or EGF signaling induces HCG-competent P(1-8).p to adopt 2° HCG-like fates. This ectopic HCG fate transformation differs from a *pry-1* phenotype in particular the HCG fate induced: a *pry-1* mutation promotes the 1° fate, whereas the combination of additional *mab-5* activity and LIN-12 or EGF signaling produces the 2° fate, suggesting a functional difference among the Wnt and LIN-12 signaling pathways in HCG fate specification.

Posterior binary equivalence group, P11/P12

In the P11/P12 equivalence group, the P12 fate is the 1° fate and the P11 fate the 2° fate (Sulston and White, 1980; Jiang and Sternberg, 1998). Normally P11/P12R adopts the 1° fate, becoming P12, and P11/P12L adopts the 2° fate, becoming P11 (Sulston and

Horvitz, 1977; Sulston and White, 1980; Jiang and Sternberg, 1998). P11/P12 fate determination depends on EGF, Wnt signaling and a posterior Hox gene *egl-5* (Herman and Horvitz, 1994; Eisenmann et al., 1998; Jiang and Sternberg, 1998; Eisenmann and Kim, 2000; Howard and Sundaram, 2002). Both EGF and Wnt signaling promote the P12 fate. Reduced EGF signaling in *let-23 egfr* and *let-60 ras* mutants, or reduced Wnt signaling in *lin-44* Wnt, *lin-17* Frizzled receptor, and *bar-1* β-catenin mutants causes a P12-to-P11 transformation (Herman and Horvitz, 1994; Eisenmann et al., 1998; Jiang and Sternberg, 1998; Eisenmann and Kim, 2000). In contrast, elevated EGF signaling by heat shock-induced LIN-3 EGF overexpression, or elevated Wnt signaling by a *pry-1* mutation results in the opposite P11-to-P12 transformation (Jiang and Sternberg, 1998; Howard and Sundaram, 2002). Genetic interactions indicate that the LIN-44/LIN-17 Wnt pathway and LIN-3/LET-23 EGF pathway act synergistically to determine the P12 fate (Jiang and Sternberg, 1998). These two pathways may function at different times during P12 specification: LIN-44 is required in late embryogenesis, whereas the LIN-3 signal is needed during the early phase of the L1 stage before P11/P12 enter the ventral cord (Jiang and Sternberg, 1998). The proposed model of P12 specification is that the LIN-44/LIN-17 pathway regulates P12 competence and that LIN-3 is the inductive signal. Interestingly, alteration of the P11/P12 fate decision caused by mutations in two downstream components of the Wnt pathway, *pry-1* and *bar-1*, is much stronger than defects in *lin-44* and *lin-17* mutants. Excess Wnt signaling in a *pry-1* mutant induces two P12, but *lin-44* overexpression has no effect on the P11/P12 decision (Jiang and Sternberg, 1998; Howard and Sundaram, 2002). *lin-44* and *lin-17* mutants have less than 30% P12-to-P11 fate transformation but *bar-1* mutants display over 90% P12-to-P11

transformation (Herman and Horvitz, 1994; Eisenmann et al., 1998; Jiang and Sternberg, 1998; Eisenmann and Kim, 2000). This phenotype is at least similar to, if not stronger than, the phenotype of *lin-44; let-23* double mutants (Jiang and Sternberg, 1998). Taken together, this suggests that another Wnt/Receptor pathway may function in parallel with LIN-3 pathway to induce the P12 fate. The Hox gene *egl-5* is a common target downstream of EGF and Wnt signaling (Chisholm, 1991; Jiang and Sternberg, 1998; Maloof et al., 1999). Overexpression of *egl-5* not only rescues the P12-to-P11 transformation of the *let-23* mutants but also causes two P12 fates in wild-type animals, suggesting that *egl-5* plays an instructive role in P12 fate determination (Jiang and Sternberg, 1998).

P11/P12 determination and male HCG patterning

In the nematode *Panagrellus redivivus,* P11/12R becomes P11 and P11/12L becomes P12, which is the opposite of *C. elegans* (Sternberg and Horvitz, 1981). Therefore, the biased P11/12 decision likely evolved from a random choice, as seen in the middle four pairs of P cells in *C. elegans*, (Sternberg, 1988). Since P1/P2 determination is also non-random, such a selection might be a by-product of abundant extracellular signaling in the head and the tail.

However, such a decision might facilitate the fate specification in the male hook sensillum competence group. The P11 lineage participates in two different processes in *C. elegans* males, first as the 2° fate in the P11 vs. P12 decision, and then its progeny P11.p as the 1° fate in HCG patterning (Sulston and White, 1980). There is only an

approximate two-hour difference from the time that P11/P12 enters the ventral cord until P11/P12 division (Sulston and Horvitz, 1977; Jiang and Sternberg, 1998). A pre-patterning event before the ventral migration would permit additional time to prepare all cellular changes required for P11 differentiation. For example, Wnt signaling is required for both P11/P12 specification (Jiang and Sternberg, 1998) and for male HCG patterning (work in the Chapter 2), and thus it is necessary to shift its role between these two. Consistent with this, in the Chapter 2, I will describe an interesting switch of *bar-1::gfp* expression between the male P11 and P12 lineages: Before P11 and P12 divide, bright *bar-1::gfp* is observed in P12 but not in P11; After P12 divides, faint expression of *bar-1::gfp* is present in both P12.a and P12.p originally but quickly disappears, and is undetectable in the P12.p daughter, P12.pa; On the other hand, male P11.p begins to express *bar-1::gfp* in the late L1 stage around the time of P12.p division, indicating the initiation of HCG patterning.

Perturbation of the P11/P12 decision changes the number of cells in the male HCG and can interfere with HCG fate specification. In *bar-1* mutants, there are four cells in the male HCG: the P11.p-like P12.p, which is caused by P12-to-P11 transformation, adopts the 1° HCG fate; P11.p therefore adopts the 2° fate; and both P10.p and P9.p adopt the 3° fate. Although the 1° P12.p and the 2° P11.p usually differentiate correctly in *bar-1* mutants, the resulting hook morphology is often abnormal due to lack of the P12.pa hyp12 epidermis and the whole hook sensillum can be mis-located anteriorly (H. Yu and P. W. Sternberg, unpublished). Moreover, a second rudimentary hook or a second HOB hook neuron, as indicated by *ceh-26::gfp* expression, or even both a second hook and a

second HOB, was occasionally observed in *bar-1* mutants (4/71) (H. Yu and P. W. Sternberg, unpublished), suggesting that P10.p adopts the 2° fate at a low frequency. Due to its more posterior position, P10.p normally receives more Wnt signal than P9.p does, which might override the lateral inhibition from a 2°-fated P11.p in *bar-1* mutants and result in a second 2° fate in a few cases. Similar hook abnormalities, including a hookless phenotype, and ectopic hook formation are also found in *egl-5* mutants (Chisholm, 1991). *mab-5*, which is the determinant of HCG competence based on my work in the Chapter 2, is expressed in P12 and its descendants P12.a and P12.p until the expression of *egl-5* in these two cells (Ferreira et al., 1999). Although *egl-5* is not expressed in P(9-11).p, its expression in the P12 lineage defines the posterior boundary of the male HCG domain. In summary, a normal P12 fate not only ensures HCG fate specification in males but also produces P12.pa, which might be required for the correct positioning and even for migration of the hook structure cell and the hook neurons.

Sex-specific organogenesis

The morphology and function of the hermaphrodite vulva and the male hook is very distinct, raising a question about how these disparate structures could arise from a similar set of signals. A major difference between VPC and HCG fate specification is the inductive signaling for the 1° fate (Fig 2). EGF is the inductive signal for the 1° VPC fate (Hill and Sternberg, 1992). My studies presented in the Chapter 2 will show that Wnt signaling promotes the 1° HCG fate. However, different signaling pathways may not be the direct cause of sexually dimorphic organogenesis. Excessive Wnt signaling can at least partially substitute for EGF signaling in VPC induction (Gleason et al., 2002),

and *vice versa* in HCG specification (work in the Chapter 2). One possible explanation is that the usage of different inductive signaling in *C. elegans* VPC and HCG patterning is based on the local abundance of the signaling: a concentrated source of signal is important for the creation of a sharp gradient to discriminate individual fates. Although there is Wnt signaling in the central region of the body (Eisenmann et al., 1998; Inoue et al., 2004) and there is EGF signaling in the tail (Chamberlin and Sternberg, 1994; Jiang and Sternberg, 1998), the EGF signal emanates from a concentrated source, the gonadal anchor cell, while Wnt signaling is more abundant in the tail region as elucidated by extensive tail defects caused by deficient Wnt signaling (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Chamberlin and Sternberg, 1995; Herman et al., 1995; Jiang and Sternberg, 1998) .

The other major difference between VPC and HCG pattern formation is sex-specific utilization of two Hox genes, *lin-39* and *mab-5* (Fig 2). Expression patterns of Hox genes are the same in both sexes, with *lin-39* expression in P(3-8).p and *mab-5* expression in P(7-11).p (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993). However, in hermaphrodites, *lin-39* function is favored, and *mab-5*'s ability to prevent P(9-11).p fusion with hyp7 is somehow blocked (Salser et al., 1993). As a transcription factor, *mab-5* regulates target gene expression. It is possible that a negative regulator in hermaphrodites sequesters *mab-5* from its targets. Alternatively, *mab-5* may act with a co-regulator that is missing in hermaphrodites. On the other hand, *lin-39* activity in males is not reinforced due to lack of a strong extrinsic signal in the central region. Interestingly, increasing different signaling in this region without alteration of Hox gene

expression pattern generates vulva-like tissue in males (Greenwald et al., 1983; Ferguson and Horvitz, 1985; P. W. Sternberg and H. R. Horvitz, unpublished), whereas additional *mab-5* expression combined with increased signaling causes hook-like fates in the same region (work in the Chapter 2). Therefore, the choice between the two Hox genes *lin-39* and *mab-5* determines whether a hermaphrodite vulva or a male hook, respectively, is formed (Maloof and Kenyon, 1998; work in the Chapter 2).

Signal integration

With similar origin and similar three-fate choices, hermaphrodite VPCs and the male HCG is a good pair for comparison of the mechanisms by which multiple signaling pathways integrate to control the generation of a precise spatial pattern of distinct cell types during organogenesis (Sulston and White, 1980; Herman, 1991; Fig 2). In general, both VPC and HCG patterning go through a three-phase process including competence, specification and execution (Sulston and White, 1980; Sternberg and Horvitz, 1986; work in the Chapter 2). During these two developmental processes, EGF and Wnt signaling provide cues from cells outside of the equivalence group (Hill and Sternberg, 1992; Katz et al., 1995; Eisenmann et al., 1998; Inoue et al., 2004; P. W. Sternberg, unpublished; work in the Chapter 2), while LIN-12 signaling transmits lateral signals among cells within the equivalence group (Greenwald et al., 1983; work in the Chapter 2).

In a closer view, both VPC and HCG competence is established by Wnt signaling (Eisenmann et al., 1998; work in the Chapter 2) and one of the two Hox genes, *lin-39* and *mab-5*, respectively (Clandinin et al., 1997; work in the Chapter 2). Different VPC and

HCG fates are specified in a stepwise manner, and in both cases, the 1° fate induces the 2° fate (Sulston and White, 1980; Sternberg and Horvitz, 1986; Herman, 1991). A graded inductive signal, which is EGF in VPC induction (Sternberg and Horvitz, 1986; Katz et al., 1995)and Wnt in HCG induction (work in the Chapter 2), respectively, causes a subset of each equivalence group to adopt induced cell fates (1° and 2°), with a stronger inductive signal stimulating the 1° fate. The 1° fate cell probably produces LIN-12 ligands that activate LIN-12 signaling in its induced neighbors to promote the 2° fate in VPC (Greenwald et al., 1983; Yochem et al., 1988; Chen and Greenwald, 2004) and HCG (Greenwald et al., 1983; work in the Chapter 2) patterning. The execution of individual 1° fates in VPC and HCG patterning likely depends on their respective inductive signals (Wang and Sternberg, 2000; work in the Chapter 2), and the execution of the 2° fates depends on LIN-12 signaling (Greenwald et al., 1983; work in the Chapter 2). In addition, a LIN-17-mediated Wnt pathway affects 1° and 2° fate execution in both hermaphrodite VPCs (Sternberg and Horvitz, 1988; Wang and Sternberg, 2000; Inoue et al., 2004) and the male HCG (work in the Chapter 2).

Unlike VPC and HCG patterning, in which only one signaling pathway plays a major inductive role, EGF and Wnt signaling share a comparable weight in P11/P12 specification, as elucidated by results of LIN-3 overexpression and mutations in *pry-1* and *bar-1* (Eisenmann et al., 1998; Jiang and Sternberg, 1998; Eisenmann and Kim, 2000). Another difference is that a different Hox gene *egl-5* is involved in P12 fate specification (Chisholm, 1991; Jiang and Sternberg, 1998). Moreover, *egl-5* is only expressed in one lineage of the binary equivalence group, and the role of *egl-5* in P12 fate

formation is instructive rather than permissive (Jiang and Sternberg, 1998; Ferreira et al., 1999). In contrast, both *lin-39* and *mab-5* play a permissive role in VPC or HCG induction, as neither multi-vulvae (Maloof and Kenyon, 1998) nor multi-hooks (work in the Chapter 2) are observed with overexpressed *lin-39* or *mab-5*, respectively. LIN-12 lateral signaling has no effect on the P11/P12 decision.

P11/P12 determination is one type of binary fate decision, in which inductive signaling plays a major role. A well-known example of this kind of binary specification is R7 photoreceptor induction in *Drosophila* (Reviewed by Greenwald and Rubin, 1992). An EGF signal from the R8 cell induces one of the five cells in an equivalence group to become R7, whereas the remaining four become cone cells surrounding the photoreceptors in an ommatidium. Another common type of binary decision, such as the AC/VU (ventral uterine precursor cell) decision in *C. elegans* gonadogenesis and neural/epidermal choices in *Drosophila* proneural clusters, is lateral specification, in which an initial variance in lateral signaling is amplified by a feedback mechanism and causes a homogeneous field of cells to become distinguished from each other (Reviewed by Sternberg, 1988; Greenwald and Rubin, 1992; Rooke and Xu, 1998). LIN-12 (Notch) signaling is a general mediator of binary lateral specification (Seydoux and Greenwald, 1989; Reviewed by Simpson, 1997). More complicated binary decisions can involve both inductive and lateral signaling. Such an example is specification of muscle and cardiac progenitors in the *Drosophila* embryonic mesoderm (Carmena et al., 1998; Halfon et al., 2000; Carmena et al., 2002). At first, Wnt signaling and Dpp TGF-β-like signaling determine a precluster in which cells are competent to respond to EGF and FGF

inductive signal (Carmena et al., 1998; Halfon et al., 2000). The localized inductive signal induces a subset of precluster cells to form an equivalence group (Carmena et al., 1998; Halfon et al., 2000). Finally, lateral Notch signaling allows one cell to be singled out from the equivalence group to become the progenitor cell (Carmena et al., 2002). This scheme is very similar to VPC and HCG patterning: the precise cell fate is generated by progressive restriction through competence, induction, and lateral inhibition mediated by multiple signal integration at different steps, representing a general scenario of complex pattern formation.

Terminal differentiation and biological function of the *C. elegans* male HCG

Cell fate execution not only involves the completion of a correct cell division pattern, but also refers to the final cell identities of the progeny including terminal morphologies and biological functions. Right after the P(10-11).p divisions, most progenies lie in a row anterior-posteriorly at the ventral side. During L4 male tail remodeling, those cells change their shapes and positions dramatically (Sulston et al., 1980). The hook and its supporting cells migrate posteriorly to reside at just anterior of the cloaca and complete the final morphogenesis to form a hook-shaped sclerotic structure. The hook sensillum mediates the step of vulval location during mating (Liu and Sternberg, 1995). Ablation of either hook neuron (HOA or HOB) abolishes normal vulva location behavior (Liu and Sternberg, 1995), suggesting that the two hook neurons work in concert for efficient vulva location. A critical question for terminal differentiation of the hook is how the presumptive HOA and HOB cells from the P10.p lineage assume the correct neuronal fate and what are the functional specificities of HOA and HOB. In the Chapter 3, I will

describe a genetic screen I conducted to identify candidates that affect the HOB fate based on expression of an HOB marker, *ceh-26::gfp* and several mutants isolated from this screen. In the same chapter, I will show that further examination of one of these mutants, *sy628*, reveals a regulatory cascade, including transcription factors *egl-46* (Wu et al., 2001), *egl-44* (Wu et al., 2001), and *daf-19* (Swoboda et al., 2000), during specification of HOB sensory function. More detailed analysis of HOB and HOA differentiation will provide some insights into the mechanisms involved in the late phase of hook development.

Figure 1. Twelve *C. elegans* Pn.p cells. Six central Pn.p cells, P(3-8).p, are vulval precursor cells (VPCs) in hermaphrodites. In males, three posterior Pn.p cells, P(9-11).p, form the hook sensillum competence group (HCG).

Figure 1

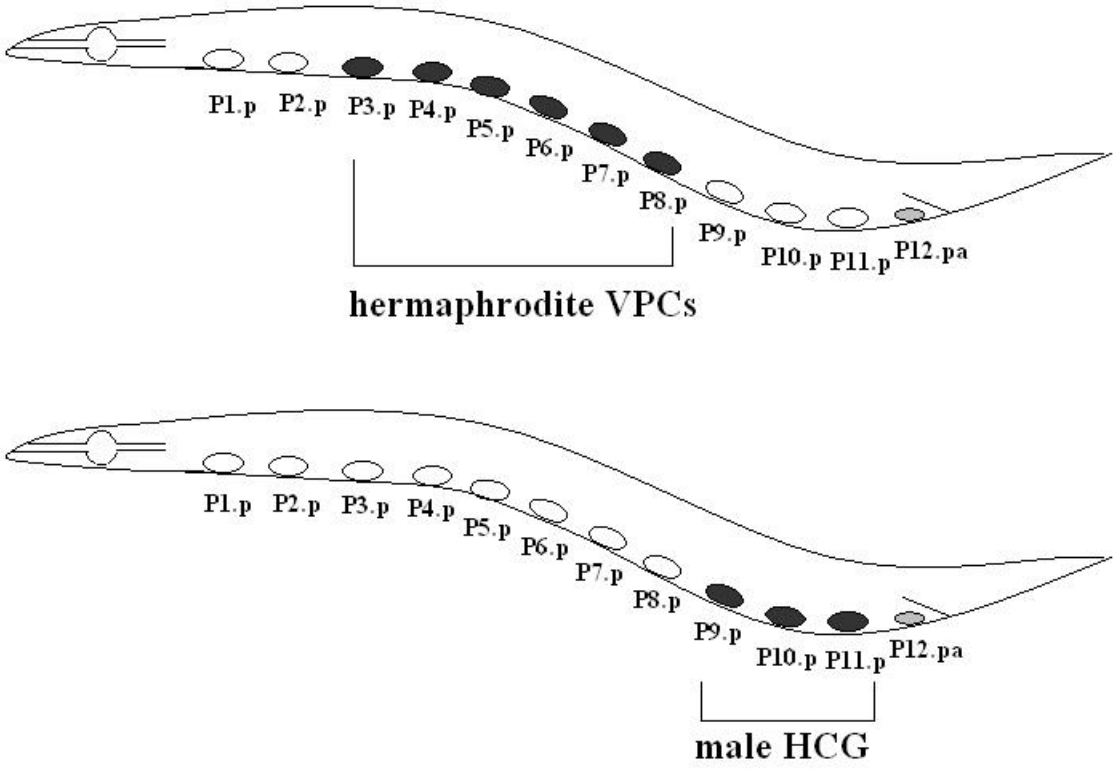
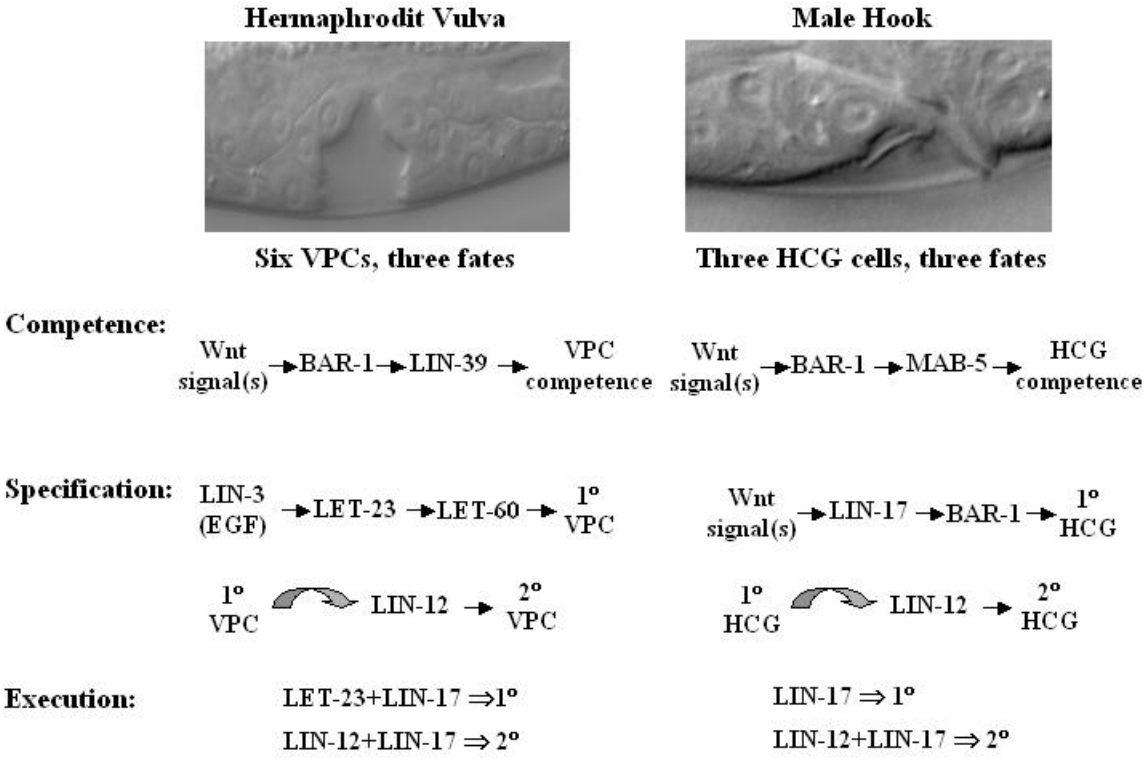

P1.p
P2.p
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
P9.p
P10.p
P11.p
P12.pa
hermaphrodite VPCs
P1.p
P2.p
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
P9.p
P10.p
P11.p
P12.pa
male HCG

Figure 2. Signaling involved in VPC and HCG patterning. Both VPCs and HCG have three potential cell fates (1°, 2°, or 3°). The precise spatial pattern of fates of each group is established by integration of multiple signaling pathways during three determinative steps, competence, specification, and execution.

Figure 2


Hermaphrodit Vulva
Male Hook
Six VPCs, three fates
Three HCG cells, three fates
Competence:
Wnt signal(s) → BAR-1 → LIN-39 → VPC competence
Wnt signal(s) → BAR-1 → MAB-5 → HCG competence
Specification:
LIN-3 (EGF) → LET-23 → LET-60 → 1° VPC
Wnt signal(s) → LIN-17 → BAR-1 → 1° HCG
1° VPC → LIN-12 → 2° VPC
1° HCG → LIN-12 → 2° HCG
Execution:
LET-23+LIN-17 ⇒1°
LIN-12+LIN-17 ⇒ 2°
LIN-17 ⇒ 1°
LIN-12+LIN-17 ⇒ 2°

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CHAPTER 2

Patterning of *C. elegans* hook sensillum lineages by Wnt signaling, Hox, and LIN-12

Abstract

Nematode ventral epidermal precursors display position- and sex-specific cell fates. Three multipotent precursor cells, P(9-11).p, form the *C. elegans* male hook sensillum competence group (HCG). Like hermaphrodite vulval precursor cells (VPCs), each HCG cell chooses one of three fates (1°, 2°, 3°), and LIN-12 signaling is necessary for 2° fate specification. We examined specification of cell fates in *C. elegans* male HCG and found that the competence of HCG cells depends on Wnt signaling and the Hox gene *mab-5*. In contrast to vulval development in which EGF signaling induces the 1° VPC fate, a *bar-1* β-catenin pathway of Wnt signaling promotes the 1° fate in the HCG. Activation of the *bar-1* pathway by decreased Axin alters the competence of anterior P(3-8).p cells and induces ectopic 1° HCG fates. This recruitment of P(3-8).p to the HCG by excess Wnt signaling requires *mab-5* activity. Misexpression of *mab-5* in P(1-8).p is sufficient to provide these anterior Pn.p cells with HCG competence, and causes formation of ectopic HCG fates in combination with activated LIN-12 or EGF signaling. In addition, genetic interactions among *lin-17* (which encodes a Wnt-receptor), *mab-5*, and *lin-12* indicate that Frizzled-type receptor-mediated Wnt signaling is necessary for HCG fate execution.

Introduction

Development of multicellular organisms often involves differentiation of fates among a set of multipotent cells called an equivalence group. Cells of an equivalence group possess similar developmental potentials but adopt different fates due to cell-cell interactions. In *C. elegans,* twelve Pn.p cells, P(1-12).p, locate ventrally along the anterior-posterior axis and generate two sex-specific equivalence groups (Sulston and Horvitz, 1977; Sulston and White, 1980). In hermaphrodites, six central Pn.p cells, P(3-8).p, form the well-known vulval equivalence group (Sulston and White, 1980; Kimble, 1981; Sternberg, 1988a). Three posterior Pn.p cells, P(9-11).p, form a different equivalence group in males, which we refer to as the hook sensillum competence group (HCG) (Sulston and White, 1980; Herman, 1991). Both groups have three different cell fates (1°, 2°, 3°), of which hierarchies are identified by the replacement after cell ablations. The Pn.p cells outside either equivalence group fuse with the hyp7 syncytial epidermis. The sex-specific fusion pattern of Pn.p cells is established by regional activities of HOM-C genes (Salser et al., 1993). Three members of a HOM-C pseudo-cluster, *lin-39, mab-5,* and *egl-5,* are expressed in distinct sets of Pn.p cells during early development. The expression domain of *lin-39* is from P3.p to P8.p (Clark et al., 1993; Wang et al., 1993), whereas *mab-5* is expressed in P7.p to P11.p (Salser et al., 1993; Wang et al., 1993). *egl-5* is expressed in P12.p and its daughter P12.pa (Jiang and Sternberg, 1998; Ferreira et al., 1999) and represses *mab-5* expression in these two cells (Salser et al., 1993). In hermaphrodites, *lin-39* activity in P(3-8).p keeps these cells unfused and competent to form VPCs, while other Pn.p cells fuse with hyp7 in the L1 stage (Clark et al., 1993; Wang et al., 1993). In males, either *lin-39* or *mab-5* activity

alone prevents cell fusion, but co-expression of *lin-39* and *mab-5* in the same cell neutralizes each other's activity. As a consequence, P(1-2).p cells, which express none of these three HOM-C genes, and P(7-8).p cells, which express both *lin-39* and *mab-5*, fuse in the late L1 stage; while P(3-6).p and P(9-11).p remain unfused at the same stage (Salser et al., 1993). The HCG cells, P(9-11).p, correspond to a domain with only *mab-5* activity (Salser et al., 1993; Maloof and Kenyon, 1998). A *mab-5* loss-of-function mutation completely eliminates this equivalence group by causing the fusion of P(9-11).p cells with hyp7 in late L1 (Kenyon, 1986).

Hermaphrodite vulval precursor cells (VPCs), P(3-8).p, are able to adopt any one of three cell fates, but exhibit a specific fate pattern of 3°-3°-2°-1°-2°-3° in response to an inductive signal from the gonadal anchor cell (Sternberg and Horvitz, 1986). P6.p, the most proximate Pn.p to the anchor cell, becomes a 1° cell, P5.p and P7.p become 2° cells, and the more distant P3.p, P4.p, and P8.p become 3° cells. The 1° P6.p and the 2° P5.p and P7.p cells go through multiple rounds of cell divisions, giving rise to the cells that form the vulva in adult hermaphrodites. The inductive signal graded from the anchor cell is LIN-3 (EGF), which then activates the LET-23 (EGFR) signaling pathway in P6.p, specifying the 1° vulval fate (Sternberg and Horvitz, 1989). A Notch homolog LIN-12 mediates a lateral signal to induce the 2° fate in VPCs (Greenwald et al., 1983; Ferguson et al., 1987; Sternberg and Horvitz, 1989; Yoo et al., 2004). *lin-15*, which encodes two negative regulators of the *let-23* pathway, is required for the 3° fate during vulval development (Sternberg, 1988b; Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994). In addition, vulval abnormalities are also observed in mutants defective in

components of Wnt signaling, including the Wnt receptor gene *lin-17* (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Wang and Sternberg, 2000; Inoue et al., 2004), the Axin homolog gene *pry-1* (Gleason et al., 2002), the APC-related gene *apr-1* (Hoier et al., 2000), and the β-catenin protein gene *bar-1* (Eisenmann et al., 1998; Eisenmann and Kim, 2000).

In the male HCG, P10.p and P11.p divide multiple times from the middle to the late third larval (L3) stage, about the same time of hermaphrodite VPC cell divisions (Sulston and Horvitz, 1977). The descendents of the P10.p and P11.p cells have distinct cell fates, some of which participate in the formation of the hook sensillum, a male copulatory structure involved in vulva location behavior during mating (Sulston et al., 1980; Liu and Sternberg, 1995). Lineage and electron microscopical reconstruction by Sulston *et al.* (1980) demonstrate that major components of the hook sensillum, including a hook structure cell, two supporting cells (hook socket cell and sheath cell), and two hook sensory neurons (HOA and HOB), are produced by the P10.p lineage. Four anterior granddaughters of P11.p are neuronal cells in the preanal ganglion, and the three posterior P11.p progeny are hypodermal cells associated with the hook sensillum. P9.p usually does not divide, while in case both P11.p and P10.p are removed by laser ablation, P9.p can adopt the P11.p fate (Sulston and White, 1980; Herman, 1991). If just P11.p is ablated, P10.p can replace the missing P11.p fate and P9.p can assume the normal P10.p fate. This anterior-to-posterior direction of recruitment after cell ablation designates the P11.p fate as the primary fate (1°), P10.p as the secondary fate (2°), and P9.p as the tertiary fate (3°) (Sulston and White, 1980). Therefore, wild-type male P(9-11).p exhibit

an invariant fate pattern of 3°-2°-1°. Signals required for specification of male P(9-11).p HCG fates remain a mystery. Extensive cell killing in the tail region has so far failed to locate the source of inductive signals for this group (Sulston and White, 1980; Herman, 1991). One known requirement is the LIN-12 pathway, which promotes the 2° fate in the HCG, a role similar to its function in the vulva (Greenwald et al., 1983). Normally, formation of the 2° fate depends on the presence of a 1° fate, however, a gain-of-function mutation of *lin-12* gene causes male P(9-11).p cells to adopt 2° fates, generating one or two ectopic 2° hooks (Greenwald et al., 1983).

The pattern formation within the male hook sensillum competence group can be divided into three different stages: establishment of HCG competence, HCG fate specification, and HCG fate execution. Here we demonstrate that Wnt signaling functions in all three steps of HCG patterning. Both the *lin-17* Frizzled-like Wnt receptor and *bar-1* β-catenin are preferentially expressed in the 1°-fated P11.p cell, suggesting a role for Wnt signaling in specification of the 1° P11.p fate. In P11.p, subcellular localization of a BAR-1::GFP fusion protein changes during the middle to late L2 stage, suggesting a time window critical for 1° fate specification. Activated Wnt signaling by a *pry-1* mutation not only changes the competence of P(3-8).p but also produces ectopic 1° HCG fates in those anterior Pn.p cells. Downstream of Wnt signaling, the Hox gene *mab-5* is the determinative element for HCG competence. Additional *mab-5* activity, plus a proliferation signal from a non-Wnt pathway, mimics the effect of Wnt signaling hyperactivity to induce HCG-like fate transformations in anterior Pn.p cells (P(1-8).p). However, neither a *mab-5* gain-of-function mutation nor the activated LIN-12 signaling

bypasses the requirement of wild-type LIN-17 function for HCG patterning. Our findings indicate that Wnt signaling plays more than one role during HCG pattern formation: The first role is to establish HCG competence; the next is to specify the 1° HCG fate, and a third is to ensure HCG fate execution.

Results

Morphological and molecular markers of the male hook sensillum competence group

The *C. elegans* male hook sensillum competence group comprises three multipotent Pn.p cells, P(9-11).p . In wild-type males, P(9-11).p each adopt one of the three potential fates (1°, 2°, or 3°), forming an invariant spatial pattern of 3°-2°-1° (Fig 1A; Sulston and White, 1980). These different HCG fates can be distinguished by individual cell division patterns and the types of progeny produced. All major components of the hook sensillum descend from the 2° P10.p cell (Sulston et al., 1980). The presence of a 1° HCG fate is necessary for a 2° fate to be expressed (Herman, 1991). To uncover the mechanism of HCG fate specification, we utilized both morphological features and the expression of lineage-specific genes to identify the 1° and 2° HCG fates in different mutants.

Upon the completion of cell divisions during the late L3 stage or at the L3 lethargus, all nine progeny of P10.p and three posterior progeny of P11.p align longitudinally at the ventral midline anterior to the anus. The four anterior offspring of P11.p are in slightly lateral planes, and become neurons of the preanal ganglion (Sulston et al., 1980). During the L4 stage, the hook structure cell, P10.papp, migrates posteriorly and forms an

invagination just anterior to the anus. Unlike the vulval invagination, a hook invagination is relatively shallow but has an anchor-like internal structure when viewed in saggital section (Fig 1B). In adults, the hook is an arrowhead-shaped sclerotic structure with yellowish autofluorescence (Fig 1C; Sulston et al., 1980; Sulston and White, 1980). The three posterior P11.p progeny, which become hypodermal cells associated with the hook sensillum (Sulston et al., 1980), contribute to the final hook morphology. Based on our observations, these three posterior 1° progeny on their own can make a shallow, rudimentary invagination in the late L4 stage if located ectopically, and form presumably sclerotized cuticle with yellowish autofluorescence at the cloaca of adult males. Although both the 1° and 2° HCG fates are able to make some invaginations and form structures that are autofluorescent, the typical anchor-shaped hook invagination or arrowheaded hook structure is a signature of the 2° HCG fate (Sulston and White, 1980; Greenwald et al., 1983).

In addition to these characteristic morphological features, the expression of several genes is also indicators of the 1° and 2° HCG lineages. *eat-4* encodes a sodium-dependent phosphate cotransporter necessary for glutamatergic transmission (Lee et al., 1999). A single neuron anterior to the anus (or the cloaca in mature adults) expresses an *eat-4::gfp* reporter construct beginning in late L4 and continuing throughout adulthood. Based on both the cell position and cell ablation results, we determined the identity of this cell as the PVV motor neuron (P11.paaa), one of the four anterior descents of P11.p (Fig 1D, E), and therefore *eat-4::gfp* is a 1° lineage marker. A homeodomain protein gene *ceh-26* is expressed in the HOB hook neuron, whereas expression of a cilium structure gene *osm-6*

is present in both HOA and HOB hook neurons (Collet et al., 1998; Yu et al., 2003). Both *ceh-26::gfp* and *osm-6::gfp* are 2° lineage markers.

LIN-17-mediated Wnt signaling affects HCG fate execution

Wnt signaling participates in multiple developmental processes in the male tail (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Chamberlin and Sternberg, 1995; Herman et al., 1995; Jiang and Sternberg, 1998). Mutations in *lin-17*, encoding a Frizzled-like Wnt receptor, result in extensive tail defects, including deficient hook development (Sternberg and Horvitz, 1988). Cell divisions of P10.p and P11.p are abnormal and daughters appear to produce similar types of progeny cells in *lin-17* mutant males. To further characterize the effect of a *lin-17* mutation on HCG development, we examined the expression of lineage markers in *lin-17(n671)* males. The *n671* mutation truncates LIN-17 before the seventh transmembrane domain, and likely eliminates gene activity (Sawa et al., 1996). We found that 89% of *lin-17(n671)* animals lacked 1° PVV expression of *eat-4::gfp* (Table 1). The remaining 11% had two to four *eat-4::gfp*-expressing cells in proximity, indicating an abnormal 1° fate. In the same strain, 7% of males formed a 2° hook-like protrusion with autofluorescence at a position corresponding to P10.papp (the hook structure cell) before its posterior migration. Only 2 of 110 animals had both an *eat-4::gfp*-expressing cell and an abnormal hook, indicating a low incidence of differentiation of both 1° and 2° lineages. In *lin-17* mutants, the expression of a 2° marker *ceh-26::gfp* was absent in all animals, and expression of *osm-6::gfp* was absent in most animals (Table 1). We infer that progeny of P10.p and P11.p in *lin-17* mutants differentiate incorrectly and generally fail to express features of HCG fates. It has been

suggested previously that *lin-17* might function in each cell division to maintain correct cell polarity during HCG fate execution (Sternberg and Horvitz, 1988; Sawa et al., 1996).

LIN-12 activity promotes a 2° HCG fate. A gain-of-function mutation of *lin-12* bypasses the requirement of a 1° cell fate and enables all three cells of P(9-11).p to adopt a 2° fate, forming up to three hook sensilla (Greenwald et al., 1983). We tested if the HCG defect caused by a *lin-17* mutation can be rescued by forced LIN-12 signaling. We found that most adult males homozygous for the *lin-17(n671)* mutation and carrying at least one copy of the *lin-12(gf)* allele had a Lin-17-like phenotype (Table 1). Fewer than one-third (31/94 and 28/135, respectively) of males had a 2° hook structure at the position of P10.p or P9.p, occasionally accompanied by hook neuron(s) (7/28). The frequency of *eat-4::gfp*-expressing cells in the double mutants was not obviously different from that in *lin-17* single mutants (Table 1). When we examined the same *lin-17; lin-12(gf)/lin-12(lf)* strain in the L4 stage, we found P9.p had proliferated in 22/29 cases, as opposed to remaining undivided in *lin-17* single mutants. Thus, reduced Wnt signaling in *lin-17* mutants suppresses the multi-hook phenotype of the *lin-12(gf)* mutation but does not block the proliferation of P9.p caused by activated LIN-12 signaling, consistent with a role of LIN-17 in HCG fate execution. However, we cannot rule out that a LIN-17-mediated pathway participates in the other steps of HCG patterning.

Expression of components of Wnt pathway marks the primary fate of P11.p

Sawa et al (1996) observed that *lin-17* is expressed in male P(10-11).p lineages. Using an extrachromosomal array carrying a GFP reporter under the control of the *lin-17* 5'

regulatory region, we observed a similar expression pattern. Before P10.p and P11.p divide, *lin-17::gfp* is expressed predominantly in the 1° P11.p cell and is barely detectable in P10.p (Fig 2B). No expression is detected in P9.p. The spatially graded expression of a Wnt receptor among P(9-11).p cells could indicate difference in competence to respond to a Wnt signal. The difference may also indicate a response to a graded Wnt signal, for example, if the receptor is up- or down-regulated by Wnt signaling. After the HCG cell divisions, both P10.p and P11.p progeny express *lin-17::gfp* with relatively higher expression in P11.p progeny (Fig 2D)

To assess whether Wnt pathways are activated in P10.p and P11.p, we analyzed the expression pattern of *bar-1*(β-catenin), a downstream component in the canonical Wnt pathway. The *gaIs45* transgene contains a rescuing *bar-1::gfp* construct (Eisenmann et al., 1998); therefore, expression of this GFP fusion protein should reflect the cellular behavior of native BAR-1 protein. Faint, cytoplasmic-dominant expression of *bar-1::gfp* is sometimes seen in P9.p up to the middle L2 stage. In P10.p, *bar-1::gfp* expression is undetectable before the cell division, but became visible in the nucleus of the posterior daughter, P10.pp. Expression of *bar-1::gfp* first appears in P11.p in the late L1 stage shortly after the birth of Pn.p cells and increases during the L2 stage (Fig 3A-F). In the early to middle L2 stage, BAR-1::GFP accumulates in the cytoplasm of the P11.p cell as puncta (Fig 3F), probably resulting from stabilization of BAR-1 proteins by releasing them from GSK3β/Axin(PRY-1)/APC(APR-1) degradation complex in response to increased Wnt signaling. From the middle to late L2 stage, bright punctate GFP fluorescence in the cytoplasm rapidly decreases (Fig 3H). By the L3 stage, right before

the first division of P11.p, BAR-1::GFP expression is predominantly nuclear (Fig 3J). After the first round of cell divisions, faint nuclear expression of BAR-1::GFP seems present in both P11.p daughters but is slightly more in the posterior one (P11.pp). The loss of cytoplasmic GFP accumulation could be due to overall decreased *bar-1::gfp* expression and longer perdurance of BAR-1::GFP proteins in the nucleus. It is also possible that nuclear translocation of BAR-1 proteins is increased during the late L2 stage, which transduces alterations in intercellular Wnt signaling into the nucleus to regulate expression of downstream target genes necessary for P11.p fate determination. The dynamic pattern of *bar-1::gfp* expression inside of P11.p clearly indicates changes in Wnt signaling.

The enhanced expression of *lin-17* and *bar-1* in P11.p suggests a role for Wnt signaling in specification of the 1° fate. In agreement with this hypothesis, the expression of *bar-1::gfp* in male P11.p is disrupted in *lin-17* mutants. Faint, uniform GFP expression is present in some males in late L1 or early L2, however, by the early L3 stage, there is no detectable *bar-1::gfp* expression in P11.p. This might be due to elevated BAR-1 degradation in *lin-17* mutants since activated Wnt signaling is required to stabilize β-catenin proteins (reviewed by Nelson and Nusse, 2004). This Wnt pathway might have some feedback regulation on *bar-1* expression as well. In a *lin-17* mutant background, the failure to establish nuclear expression of *bar-1* by the L3 stage could be a sign of a failure to induce a 1° HCG fate in P11.p. The low-level expression of Wnt pathway components *lin-17* and *bar-1* in P9.p and P10.p could indicate the involvement of this Wnt pathway in determination of HCG competence.

Activated Wnt signaling promotes 1° HCG fates in anterior Pn.p cells

A loss-of-function mutation in *bar-1* causes a mild defect in hook formation (Table 2). Specifically, 14.1% of *bar-1(ga80)* males lacked both hook structure and hook neurons, and the other 29.6% had a partial loss of the 2° fate, with absence of either the hook structure or the hook neuron (n=71). This phenotype is less severe than the *lin-17* mutant phenotype, which might reflect redundancy of multiple Wnt sub-pathways in HCG patterning.

We next assessed the effect of Wnt pathway activation using the *pry-1(mu38)* mutation, which inactivates an Axin homolog, a negative regulator of the canonical Wnt pathway (Korswagen et al., 2002). We noticed that L4 larvae of *pry-1(mu38)* males form ventral invaginations sometimes with an anchor-like shape inside, and the adults have several small protrusions that almost all have yellowish autofluorescence under UV illumination regardless of the shape (Fig 4A-B). These morphological features resemble structures associated with HCG-like fates, developed from proliferation of Pn.p cells anterior to P(9-11).p. About 70% of *pry-1(mu38)* males yielded such a morphological alteration in P(3-6).p and about 30% in P(7-8).p (Table 2). The normal 3°-2°-1° pattern of P(9-11).p cell fates is disrupted in the *pry-1* mutant background, due to transformation of P11 to P12-like fate in the L1 stage (Howard and Sundaram, 2002). This also affects specification of the 2° P10.p fate and thus results the absence of a wild-type hook in the cloacal region. HCG-like invaginations and protrusions from P(9-10).p were observed at low frequency (Table 2).

In wild-type males, although P(3-6).p remain unfused in the L1 stage as P(9-11).p do, they are not competent to adopt HCG fates and normally fuse with hyp7 during late L2 (Sulston and White, 1980; see Appendix of the Chapter 2). In response to activated LIN-12 or EGF signaling, male P(3-6).p can escape the fusion fate in L2 and assume vulval-like fate, producing pseudo-vulvae at the ventral side (Greenwald et al., 1983; Ferguson and Horvitz, 1985; P. W. Sternberg and H. R. Horvitz, unpublished). As the shape of an ectopic hook-like invagination or protrusion is often irregular, it is not easily distinguishable from structures generated by a vulval-like fate in some cases. In *pry-1* mutant males, the central Pn.p cells including P(3-6).p and P(7-8).p were usually unfused as visualized by *ajm-1::gfp,* a marker for cell fusion, and some of these cells divided multiple times during the late L3 to early L4 stage. To clarify if a *pry-1* mutation causes HCG fate transformation (as opposed to ectopic vulval differentiation) among the central Pn.p cells, we examined the expression of lineage-specific markers in *pry-1* mutant males. No expression of *egl-17::cfp,* a vulval fate marker, was detected at the ventral protrusions of *pry-1* mutant males. Strikingly, *pry-1* mutants produced many *eat-4::gfp*-expressing ventral neurons with processes similar to PVV, each frequently associated with a ventral protrusion (Fig 4E-F; Table 2). This expression of a 1° HCG lineage marker as well as morphological features of ventral structures suggests that the *pry-1* mutation not only prevents fusion of the anterior P(3-8).p cells with hyp7 but also changes the competence of these cells and promotes ectopic 1° HCG fates among now-competent anterior Pn.p cells.

Although some protrusions in *pry-1(mu38)* males, by both marker expression and morphology, resemble ectopic 1° HCG fate, the structure of others resembles the 2° hook, which could be induced by a nearby 1° Pn.p cell. Alternatively, some 1°/2° hybrid fates are formed in the *pry-1(mu38)* genetic background. Presence of ectopic hook neurons, indicated by 2° marker expression, is less frequent than occurrence of the hook-like structure. Expression of two 2° hook neuron markers, *ceh-26::gfp* and *osm-6::gfp,* was occasionally observed adjacent to a ventral protrusion in the *pry-1* mutant background (Fig 4C-D; Table 2), indicating that differentiation of hook neurons perhaps requires more intricate interactions.

Specification of extra HCG fates in *pry-1* mutants is fully suppressed by a *bar-1* mutation (Table 2). No anterior invaginations or protrusions were found in *pry-1; bar-1* double mutants, and 2° hook structures and neurons were only derived from P(9-11).p, the normal HCG region (n= 147). The hook sensillum was completely absent in 6.8% of double mutants, and was partially lost in another 10.9% (n= 147), indicating a weak defect in 2° lineage specification similar to what is seen in a *bar-1* single mutant. This suppression suggests that the aberrantly activated canonical Wnt signaling pathway accounts for establishment of HCG competence in P(3-8).p cells and production of ectopic 1° fates in *pry-1* mutants.

Male P(3-8).p adopt vulval-like fates in *lin-12(gf)* mutant males, in contrast the formation of multiple hooks from P(9-11).p in the same genetic background. After introducing a *pry-1* mutation into a *lin-12(gf)* background, additional ectopic hook neurons, visualized

by *osm-6::gfp* in the L4 stage and paired with well-formed ectopic hook invaginations, were observed among the central P(3-8).p cells of *pry-1(mu38); lin-12(gf)/lin-12(lf)* double mutants, revealing increased production of 2° HCG fates (Fig 5, Table 2). In addition, we observed ectopic hook neurons and invaginations from P(1-2).p (Table 2), a phenotype not detected in either of the single mutants. One explanation is that LIN-12 signaling enhances the formation of ectopic HCG fates in P(1-2).p caused by increased Wnt signaling. In conclusion, the activated Wnt signaling converted the anterior Pn.p cells (most frequently P(3-6).p) to HCG fates, and LIN-12 signaling reinforces 2° fate formation in cells with HCG competence. These results also indicate that LIN-12 signaling itself, unlike Wnt signaling, has little or no effect on HCG competence.

Mutation in *pry-1* acts through Hox genes to alter cell competence among anterior Pn.p cells

P(7-8).p are often unfused in *pry-1(mu38)* males, suggesting that additional Wnt signaling affects the L1 fusion decision. The mechanism of male P(7-8).p fusion relies on relative ratios of activities of two Hox genes *lin-39* and *mab-5* (Salser et al., 1993), raising the possibility that activation of the Wnt pathway in *pry-1* mutants increases *mab-5* expression, as seen in specification of Q neuroblast lineages (Maloof et al., 1999; Korswagen et al., 2002), and therefore shifts the balance of antagonistic interactions between the two Hox genes in P(7-8).p to block cell fusion. Moreover, alteration in Hox gene activities could change the competence of P(3-8).p cells. The wild-type HCG domain is from P9.p to P11.p, corresponding to a region with only *mab-5* expression.

Maloof and Kenyon (1998) speculated that generation of HCG competence depends on *mab-5* function.

To determine whether *mab-5* acts downstream of *pry-1* to specify HCG competence in P(3-8).p, we examined phenotypes of *pry-1(mu38); mab-5(e1239)* males. The *mab-5(e1239)* mutation alone causes male P(7-8).p to stay unfused as P(3-6).p do until the late L2 stage but P(9-11).p fuse with hyp7 in the L1 stage. In *pry-1(mu38); mab-5(e1239)* double mutants, male P(9-10).p frequently fuse during the L1 stage, but P(3-8).p often remain unfused until the L4 stage and in some cases divide. However, ectopic expression of HCG-like fates in *pry-1* mutants is fully abolished by a *mab-5* loss-of-function mutation (Table 3). Thus, activated Wnt signaling in *pry-1* mutants requires *mab-5* activity to express HCG fates in Pn.p cells. We propose that a mutation of *pry-1* causes *mab-5* misexpression in normally unfused P(3-6).p during the L1 and L2 stage, thereby establishing HCG competence in those cells. The additional *mab-5* expression also blocks cell fusion in P(7-8).p and makes unfused P(7-8).p cells HCG competent.

A mutation of *lin-39* causes only a regional suppression on HCG induction in *pry-1* mutant males (Table 3). The increased *mab-5* activity in a *pry-1* mutant does not override the requirement of *lin-39* function for prevention of P(3-6).p fusion in the L1 stage. In most *pry-1(mu38); lin-39(n1760)* males, P(3-6).p fuse with hyp7, and therefore greatly reduce the formation of hook-like protrusions and ectopic expression of the 1° marker *eat-4::gfp* among P(3-6).p. However, the occurrence of HCG fates in P(7-10).p

or in occasional unfused P(3-6).p is not suppressed by loss of *lin-39* activity (Table 3), suggesting that *lin-39* affects cell fusion but has no effect on HCG competence.

MAB-5 is required for more than preventing fusion in the L1 stage

In wild-type males, *mab-5* activity represses the fusion fate of P(9-11).p in the L1 stage, raising the possibility that HCG competence could merely be a direct consequence of the prevention of cell fusion. To test this hypothesis, we used an *eff-1* mutation to block P(9-11).p cell fusion in *mab-5(e1239)* mutants. *hy21* is a temperature-sensitive allele of *eff-1*, an integral membrane protein gene necessary for epithelial cell fusion (Mohler et al., 2002). When grown at 25°C, all hypodermal cells fail to fuse in *eff-1(hy21)* mutants. From our observations, *eff-1(hy21)* males exhibited a weak abnormality in hook morphology, while expression of *eat-4::gfp* in PVV was generally not affected (48/48), suggesting that HCG patterning is not changed. Although unfused P10.p and/or P11.p cells were observed in *eff-1(hy21); mab-5(e1239)* males, neither 1° expression of *eat-4::gfp* nor formation of hook structure was detected in the double mutant (n=78). In the absence of *mab-5* activity, the inhibition of cell fusion is insufficient for P(9-11).p to adopt HCG fates.

Despite their hookless phenotype and abnormal HCG lineages, *lin-17* mutations usually do not abolish cell divisions in P10.p and P11.p, raising the question of whether *mab-5* overexpression is able to rescue the lineage defects in *lin-17* mutants. To address this issue, we used the *mab-5* promoter mutation *e1751* to constitutively express *mab-5* in the Pn.p cells of *lin-17* mutants. The pattern of male Pn.p fusion in the L1 stage is altered in

mab-5(e1751) mutants: P(1-2).p remain unfused by extra *mab-5* activity but P(3-8).p fuse with the syncytial epidermis due to functional antagonism between *lin-39* and *mab-5* (Salser et al., 1993). However, no alteration in hook sensillum lineages was found in a *mab-5(e1751)* single mutant (Table 4; data not shown). All 28 *lin-17(n671); mab-5(e1751)* males examined displayed a Lin-17-like tail phenotype: 27 animals were hookless and only one had a rudimentary hook-like protrusion. Thus *mab-5(e1751)* is not sufficient to bypass the requirement of LIN-17-mediated Wnt signaling for HCG patterning. As with *lin-39* in vulval development, *mab-5* could function as a permissive signal and endow HCG competence to male P(9-11).p, but other downstream components of the Wnt pathway act in 1° fate specification and HCG fate execution. Alternatively, *mab-5* overexpression by the *e1751* mutation still depends on *lin-17*-mediated Wnt signaling; thus, it remains to be determined whether *lin-17*-independent *mab-5* activity is sufficient to rescue *lin-17* mutant defects.

Recruitment of anterior Pn.p cells to the HCG by cooperative interaction between *mab-5* overexpression and a proliferation signal

We showed that *mab-5* is not only required for prevention of P(9-11).p fusion with hyp7 but is also a key component downstream of Wnt signaling necessary for HCG competence. Wnt signaling might also act as a proliferation signal. We tested whether the combined effect of the *mab-5(e1751gf)* mutation and a non-Wnt proliferation signal could mimic the *pry-1* mutant phenotype to generate an ectopic hook sensillum among P(1-8).p.

The first combination we tried was *mab-5* overexpression and constitutively activated LIN-12 (see Materials and Methods). We found that 39% of *mab-5(e1751)+/+lin-12(gf)* males had some hook sensillum lineages in P(3-8).p and about 54% formed ectopic 2° HCG fates in P(1-2).p (Fig 6A, B; Table 4). Therefore, *mab-5* and activated LIN-12 cooperate to transform anterior P(1-8).p to adopt a 2° HCG fate. No ectopic 1° *eat-4::gfp* expression from anterior Pn.p was detected in this genetic combination (data not shown), reflecting a functional distinction of Wnt and LIN-12 signaling during HCG pattern formation. Furthermore, production of ectopic 2° HCG fates in *mab-5(e1751)+/+lin-12(gf)* males still depended on wild-type *lin-17* function: introduction of a *lin-17(n671)* mutation to the *mab-5(e1751)+/+lin-12(gf)* strain suppressed 2° fate formation in all Pn.p cells, consistent with a role of *lin-17* in HCG fate execution.

The second combination we tried was the *mab-5(e1751)* mutation with a loss-of-function mutation of the *lin-15* gene, *e1763*. Mutations in *lin-15* activate the LET-23(EGFR) pathway (Huang et al., 1994), the central signaling pathway inducing vulval development. Activated EGF signaling in a *lin-15* mutant causes P9.p to adopt a 2°-like fate, forming an ectopic rudimentary hook without additional hook neurons (P. W. Sternberg, unpublished). Those *lin-15(e1763)* males also made pseudovulvae at P(3-8).p. The male P(3-8).p Muv phenotype of a *lin-15* mutant is fully suppressed by the *mab-5(e1751gf)* mutation. About 10-20% of *mab-5(e1751gf); lin-15(e1763)* double mutants had HCG-like fates from male P(3-8).p, usually either P3.p or P8.p (Table 5). In addition, P(1-2).p cells of most double mutants not only divided, but also differentiated to make hook-like invaginations and protrusions (Fig 6C-E; Table 5). Occasionally there is a complete 2°

fate transformation as hook neurons were also detected based on ectopic *osm-6::gfp* expression (data not shown). The generation of P(1-2).p ectopic hooks by this *mab-5(e1751gf); lin-15* double mutant suggests that activated EGF signaling is capable of inducing HCG fates in Pn.p cells that have acquired HCG competence.

We also observed ectopic hook formation in *lin-12(gf)/lin-12(lf); lin-22(n372)* double mutants. *lin-22(+)* function inhibits *mab-5* expression in the anterior lateral epidermis (Wrischnik and Kenyon, 1997). In a *lin-22* single mutant, male P(7-8).p stay unfused in the L1 stage, which could be partly due to a weak increase of *mab-5* expression. Unlike what is seen in *mab-5(e1751gf)* mutants, male P(1-2).p of *lin-22* mutants still assume a normal fusion fate in L1. In general, proliferation of male P(3-8).p is enhanced in a *lin-12(gf)/lin-12(lf); lin-22(n372)* strain compared to a *lin-12(gf)/lin-12(lf)* genetic background (Fixsen, 1985; our observation). We found that 27% of the double mutant males produced 2° hook invaginations and hook neurons in P(7-8).p, while formation of pseudovulvae was predominant in P(3-6).p (Fig 7B, C; Table 5), indicating that prevention of cell fusion and enhanced *mab-5* activity in P(7-8).p make these two cells competent to adopt HCG fate. Unexpectedly, about 5.5% of the double mutant males formed an ectopic hook and/or hook neurons in P(1-2).p (Fig 7A; Table 5), though neither of the single mutants had unfused P(1-2).p cells. Interaction between the *lin-12(gf)* and *lin-22* reduction-of-function mutations may keep P(1-2).p unfused and cause ectopic *mab-5* expression to provide these two Pn.p cells with HCG competence.

Discussion

Previous studies of *C. elegans* vulval development revealed how extrinsic signals are integrated temporally and spatially with intrinsic factors to establish the precise fate pattern among a set of multipotent precursor cells. In this work, we extended our understanding of fate specification in another set of "equivalent" cells, the male hook sensillum competence group (HCG). Both hermaphrodite VPCs and male HCG are derived from a subset of Pn.p cells, P(3-8).p and P(9-11).p, respectively, and both have three fate choices. During development, male P(9-11).p lose plasticity and become committed to specific fates, resulting in an invariant wild-type pattern of 3°-2°-1°, from anterior to posterior. The 3° fate refers to a cell that has HCG competence but is not specified as 1° or 2°, i.e., the "ground state" fate; the 2° lineage makes the major components of the hook sensillum; and the 1° fate generates neurons of preanal ganglion group and hypodermal cells associated with the hook sensillum. The 1° fate is required for expression of a 2° fate (Herman, 1991). We showed that Wnt signaling has fundamental roles in determination of HCG competence, specification of the 1° HCG fate, and execution of the HCG fates. We further confirmed that Hox gene *mab-5* is the downstream target of the *bar-1/pry-1* Wnt pathway that is responsible for HCG cell competence. Finally, we demonstrated that increased *mab-5* activity with proliferative signals from a non-Wnt pathway can produce ectopic HCG fates in the Pn.p cells other than P(9-11).p, an effect similar to activation of the Wnt signaling pathway. In summary, we propose the following scheme for male HCG fate specification (Fig 8). Male P(9-11).p cells remain unfused in the late L1 stage by virtue of *mab-5* activity, and thereby are potentially responsive to an inductive signal. A posterior Wnt signal regulates *mab-5*

activity to determine HCG competence and initially establish a pattern in the P(9-11).p cells. In P11.p, activated LIN-17 receptor signals downstream via BAR-1 to promote the 1° fate, which produces ligands for receptor LIN-12(Notch) to promote the 2° fate in the adjacent P10.p cell.

Wnt signaling in P(9-11).p

The observation of faint *bar-1::gfp* expression in P9.p and faint *lin-17::gfp* expression in P10.p suggests that, initially, Wnt signaling is probably not restricted to the P11.p cell. It is possible that a weak activity of Wnt signaling functions after the L1 fusion in the posterior region to define HCG competence in P(9-11).p. The three different patterns of *lin-17* and *bar-1* expression among wild-type P(9-11).p might represent three different fates. Higher expression of *lin-17* and *bar-1* in P11.p is correlated with the 1° fate. In addition, a low level of nuclear-localized BAR-1::GFP was observed in P11.p since the beginning of *bar-1::gfp* expression during the late L1 stage, indicating that activation of Wnt signaling in P11.p starts as early as the late L1 stage. The weak expression of *lin-17* and undetectable *bar-1* expression in P10.p, which could be an outcome from interaction between P11.p and P10.p, corresponds to specification of the 2° fate. Presence of faint *bar-1::gfp* in P9.p of a few animals before the middle L2 stage is probably resulted from responses of a P9.p cell, when still retaining its development potentials, to a distant and therefore somehow ambiguous Wnt signal.

Wnt signaling and *mab-5* activity

The observations that extra *mab-5* activity in *pry-1* mutants alters the competence of P(3-6).p revealed a potential role of *mab-5* in HCG patterning other than preventing cell fusion. Our further analysis of *mab-5* double mutants with *eff-1, lin-12(gf),* and *lin-15* supported the hypothesis that Hox gene *mab-5* is the essential component downstream of Wnt signaling for HCG competence.

Prior to HCG pattern formation, the fusion of Pn.p cells in the late L1 stage to hyp7 is regulated by two Hox genes *lin-39* and *mab-5,* and has a direct impact on the existence of P(9-11).p precursor cells (Salser et al., 1993), e.g., a *mab-5* mutant lacks a hook structure due to the fusion of P(9-11).p to hyp7 in L1. It is unclear whether Wnt signaling is necessary for *mab-5* to prevent the L1 cell fusion. The unfused P(7-8).p cells in *pry-1(mu38)* males demonstrate that excess Wnt signaling affects this early fusion event. Preferential upregulation of *mab-5* activity in a *pry-1* mutant background could break the balanced antagonism between Hox genes *lin-39* and *mab-5* and thus enable P(7-8).p to escape the fusion fate. On the other hand, the increased *mab-5* activity in a *pry-1* mutant is not sufficient to interfere with *lin-39* function to cause abnormal fusion of P(3-6).p in the L1 stage as seen in *mab-5(e1751gf)* mutants. It is possible that P(7-8).p are sensitive to relative difference between these two Hox gene activities due to the nature of cell fusion mechanism. A similar inhibition of P(7-8).p fusion is also seen in *lin-12(gf)* and *lin-15* mutants, except that P(7-8).p adopt vulval-like fates in these two mutant backgrounds (Greenwald et al., 1983; Ferguson and Horvitz, 1985; P. W. Sternberg and H. R. Horvitz, unpublished) instead of HCG fates as they do in *pry-1* mutant males; this

difference suggests that *lin-39* activity is preferentially activated by LIN-12 and LET-23 pathways. However, both *lin-12(gf)* and *lin-15* mutants have a multi-hook phenotype within P(9-11).p, indicating that the preference for *lin-39* activation by a *lin-12(gf)* or a *lin-15* mutation can not compete with the influence from the posterior Wnt signal on *mab-5* activity.

Male HCG vs. hermaphrodite VPC

The male hook sensillum competence group shares several similarities with the hermaphrodite vulval precursor cells: (1) both groups are from a subset of Pn.p cells (Sulston and White, 1980); (2) each group displays three different fates (Sulston and White, 1980; Sternberg and Horvitz, 1986; Herman, 1991); (3) fate specification of both groups occurs during the L2 to L3 stage and first round of cell divisions both begins in the middle L3 stage (Sulston and White, 1980); (4) Hox gene activities are necessary and sufficient to keep subsets of Pn.p cells from fusion (Salser et al., 1993) and are regulated by Wnt signaling pathway to establish cell competence (Eisenmann et al., 1998; this work); (5) only the 1° and 2° fates of each group are induced to further differentiation by an inductive signal, and LIN-12 signaling promotes the 2° fate in both cases (Sulston and White, 1980; Greenwald et al., 1983; Sternberg and Horvitz, 1986; Sternberg and Horvitz, 1989; Herman, 1991).

However, although similar signaling pathways participate in both processes, the scenario used in fate specification is distinct for each group. During hermaphrodite vulval development, *lin-39* gene prevents L1 fusion and functions as the determinative factor for

VPC competence (Clandinin et al., 1997; Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002). A different Hox gene, *mab-5*, plays a similar role in HCG development (Kenyon, 1986; this work). The expression of *mab-5* in male P(9-11).p in the absence of *lin-39* and *egl-5* defines the boundary of HCG group.

One major difference between VPC and HCG fate specification is the signaling pathway that specifies the 1° fate. Vulval development uses the LET-23 pathway to specify the 1° VPC fate, and Wnt pathway has a minor role in induction (Gleason et al., 2002; reviewed by Moghal and Sternberg, 2003). By contrast, Wnt signaling is the major regulatory pathway for specification of the 1° HCG fate, while hyperactivation of the LET-23 pathway in a *lin-15* mutant can mis-specify 2° fate in P9.p (P. W. Sternberg, unpublished).

During vulval induction, the anchor cell produces a graded signal that initiates the patterning of the VPCs. However, many cells surrounding male P(9-11).p have been ablated individually or in various combinations using a laser microbeam but failed to reveal a cell or a group of cells that influences P(9-11).p fates (Sulston and White, 1980; Herman, 1991). Five Wnt-like ligand genes, *lin-44*, *egl-20*, *mom-2*, *cwn-1* and *cwn-2*, are present in *C. elegans* genome. Each has expression in some cells of the tail region (Herman et al., 1995; Whangbo and Kenyon, 1999; see Appendix of the Chapter 2). So far, mutations in neither of three Wnt genes, *lin-44*, *egl-20*, and *mom-2*, yielded defects in hook sensillum lineages (see Appendix of the Chapter 2).

Although activation of canonical Wnt pathway is sufficient to induce ectopic 1° HCG fate, the weak defect in the hook formation caused by loss of *bar-1* activity indicates that other components of Wnt signaling are involved in HCG patterning. In *C. elegans*, besides *bar-1*, the β-catenin supposed to function in the canonical Wnt pathway, there are other two β-catenins, *wrm-1* and *hmp-2*. WRM-1 interacts with LIT-1 protein kinase to regulate POP-1 phosphorylation (Rocheleau et al., 1999) and *hmp-2* is involved in cell adhesion (Costa et al., 1998; Korswagen et al., 2000). Overexpression of *wrm-1* or *hmp-2* can substitute for *bar-1* function during vulval development (Natarajan et al., 2001), therefore they might function redundantly in HCG fate specification. Zygotic RNAi of *wrm-1* and *hmp-2* cause male tail defects (Herman, 2001). In response to various Wnt-receptor interactions, other Wnt subpathways, such as the polarity pathway (Boutros et al., 1998; Adler, 2002; Strutt, 2003) and a Ca++ pathway (Kuhl et al., 2000), might have roles in HCG patterning as well.

In some other nematodes, such as *Mesorhabditis*, vulval development does not require a signal from the gonad (Sommer and Sternberg, 1994). Perhaps vulval development in these species depends on Wnt signaling via Hox genes as in the *C. elegans* HCG cells.

Materials and Methods

General methods, nomenclature and strains

Methods for handling and culturing of *C. elegans* are described by Brenner (1974). Unless otherwise noted, all experiments were performed at 20˚C. The following alleles were used in this work.

Linkage group (LG) I: *lin-17(n671)* (Ferguson and Horvitz, 1985), *pry-1(mu38)* (Maloof et al., 1999)

LG II: *eff-1(hy21)* (Mohler et al., 2002)

LG III: *dpy-19(e1259), unc-32(e189), lin-12 (n137, n676n909)* (Greenwald et al., 1983), *lin-39(n1760)* (Clark et al., 1993), *mab-5(e1239, e1751)* (Hodgkin, 1983; Hedgecock et al., 1987; Salser and Kenyon, 1992)

LG IV: *lin-22(n372)* (Horvitz et al., 1983), *him-8(e1487)* (Hodgkin et al., 1979)

LG V: *him-5(e1490)* (Hodgkin et al., 1979)

LG X: *bar-1(ga80)* (Eisenmann et al., 1998), *dpy-6(e16), unc-9(e101), lin-15(n765, e1763)*(Ferguson and Horvitz, 1985)

Integrated transgenes and extrachromosomal arrays were: (LG I) *syIs78(ajm-1::gfp)* (Gupta et al., 2003); (LG III) *chIs1200(ceh-26::gfp)* (Yu et al., 2003); (LG V) *mnIs17(osm-6::gfp)* (Collet et al., 1998), *arIs92(egl-17::cfplacZ)* (Yoo et al., 2004) ; (LG X) *adIs1240(eat-4::gfp)* (Lee et al., 1999); *gaIs45(bar-1::gfp)* (Eisenmann et al., 1998); *syEx676 (lin-17::gfp)* (B. P. Gupta and P.W. Sternberg, unpublished).

In the text, we refer to the *lin-12* gain-of-function mutation *lin-12(n137)* as " *lin-12(gf)*", and the null mutation *lin-12(n676n909)* as " *lin-12(lf)*". Unlinked double mutant strains were constructed according to standard methods (Huang and Sternberg, 1995). During some of strain constructions, genetic markers within same linkage group of one allele were used in trans to that allele to facilitate the selection.

To examine *lin-17::gfp* expression in male P(9-11).p lineages, *syEx676(lin-17::gfp)* hermaphrodites were crossed by *him-5* or *him-8* males to yield F1 males carrying the extrachromosomal array. Expression of *lin-17::gfp* in the P(9-11).p lineages is observed in 18/22 F1 males with the extrachromosomal array. There was no difference in *lin-17::gfp* expression between *him-5(e1490)/+* and *him-8(e1489)/+* males.

The original *eat-4::gfp* transgene integrated on chromosome X is linked to *n765*, a temperature-sensitive mutant allele of *lin-15*, raising a possibility that additional PVV-like cells might be due to an interactions between the *pry-1* and *lin-15* mutations (P. W. Sternberg, unpublished). To remove the *lin-15(n765)* allele linked to the *adIs1240(eat-4::gfp)* transgene, *him-5 (e1490)* V*; adIs1240 lin-15(n765ts)* X males were crossed into *dpy-6(e14) unc-9(n101)* X hermaphrodites and F2 Unc-non-Dpy animals expressing *eat-4::gfp* were selected. We re-assayed *eat-4::gfp* expression in *pry-1* males grown at 15°C, a permissive temperature for *lin-15(n765)*, and in *pry-1* mutants after removal of *lin-15(n765)* mutation from background by genetic recombination. In both conditions, *pry-1* mutants displayed significant ectopic *eat-4::gfp* expression, associated with ventral protrusions. These results suggest that *pry-1(mu38)* induces formation of ectopic PVVs from P(3-10).p cells, although we cannot rule out some minor effect from *lin-15*.

Linked double mutant of *pry-1(mu38)* mutation and *syIs78(ajm-1::gfp)* transgene, which are both located on linkage group I, was obtained by picking *pry-1(mu38)* homozygous hermaphrodites with *ajm-1::gfp* expression in a F2 population after cross. *pry-1; bar-1* double mutant males have a Bar-1-like gross morphology, and are much healthier than

pry-1 single mutant males, suggesting a complete suppression of the *pry-1(mu38)* phenotype by the *bar-1(ga80)* mutation. In this double mutant, the presence of *pry-1(mu38)* allele in the strain was verified by strain deconstruction. By contrast, in *pry-1; mab-5* double mutants, loss of *mab-5* function did not change the scrawny appearance of *pry-1* mutant animals, indicating that *mab-5* only participated in some aspects of Wnt signaling. Although small bumps are sporadically formed at the ventral side as a result of occasional proliferation of Pn.p cells, neither hook-like structure nor yellowish autofluorescence was seen in *pry-1(mu38); mab-5(e1239)* males. About one-third of *pry-1; mab-5* double mutant males had an additional neuronal cell expressing *eat-4::gfp* in the preanal ganglion region. By comparing them with *mab-5(e1239)* single mutants, we deduced that this neuron is not likely from the Pn.p lineages, and is probably differentiated from a Pn.a progeny that fails to die in the *mab-5(e1239)* loss-of-function mutant background (Kenyon, 1986). Unlike what is seen in *mab-5* single mutants, the fusion of P(9-10).p to the hyp7 epidermis is not complete in *pry-1(mu38); mab-5(e1239)* males. This could be due to additional *lin-39* activity, if excess Wnt signaling in *pry-1* mutants increased activities of both Hox genes, but with a preference for *mab-5*.

The *lin-17(n671); mab-5(e1751)* animals are very sick and the hermaphrodites usually rupture before production of any progeny. In the double mutant males, the presence of *mab-5(e1751)* mutation was verified based on gaps in their lateral alae (cuticular ridges). Since *mab-5* and *lin-12* are closely linked on linkage group III, we made a heterozygous strain, with one copy of chromosome III containing a *mab-5(e1751)* allele and a wild-type *lin-12(+)* allele, and the other copy a *mab-5(+)* allele and a *lin-12(gf)* allele. In this

compound heterozygous background, *mab-5(e1751)* single males can be distinguished by fused rays 1 and 2 with a wild-type hook. Males with more than one hook anterior to the cloaca, indicating the presence of *lin-12(gf),* were examined for HCG-like fate formation in anterior Pn.p.

Microscopy

Cell anatomy and lineages were observed in living animals using Nomarski optics as described (Sulston and Horvitz, 1977). We took advantage of B cell development as an internal reference for the developmental timing. The male-specific B blast cell has the first cell division at late L1, right after the generation of Pn.p cells. The second cell division of B lineage is in middle L2. By the late L2 stage, there are ten B progeny. These ten cells then adjust their positions and form a characteristic assembly around the proctoderm in the early L3 stage. Cells of the B lineage further divide right before P(10-11).p division in about the middle L3 stage. The appropriate position of developing male gonad is another temporal indicator. For viewing GFP expression, a Chroma Technology High Q GFP long pass filter set [450 nm excitation, 505 nm emission] was used in conventional fluorescence microscopy (Zeiss Axioskop). The same filter is also used for visualizing autofluorescence.

Cell ablations were performed with a laser microbeam system according to standard methods (Sulston and White, 1980; Avery and Horvitz, 1987). The nucleus of P11.p (late L2 or early L3) or P11.pa (middle L3) was destroyed in *him-5(e1490); adIs1240 lin-15(n765)* males and the recovered animals were inspected for adult *eat-4::gfp* expression.

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Table 1. Expression of lineage markers in male P(9-11).p

genotype[a]	markers[b]	marker expression (%)			no. of hooks in P(9-11).p	n[c]
		P9.p	P10.p	P11.p		
wild-type	*eat-4::gfp* (1°)	0	0	100	1	117
lin-17	"	0	0	10.9	0.07	110
lin-12(gf)/lin-12(lf)[d, e]	"	0	0	55.8	2.28	138
lin-17; lin-12(gf)/lin-12(lf)[d]	"	0	0	8.5	0.41	94
wild-type	*osm-6::gfp*[f] (2°)	0	100	0	1	>200[g]
lin-17	"	0	4.8	0	0.1	42
lin-12(gf)/lin-12(lf)	"	98.6	100	31.4	2.3	70
lin-17; lin-12(gf)/lin-12(lf)	"	0.7	5.9[h]	0	0.22	135
wild-type	*ceh-26::gfp* (2°)	0	100	0	1	>1000
lin-17	"	0	0	0	0.06	63

[a] Alleles used are: *lin-17(n671), lin-12(n137)* as *lin-12(gf),* and *lin-12(n676n909)* as *lin-12(lf).* All strains contain *him-5(e1490)* in the background.

[b] The integrated *eat-4::gfp*, *osm-6::gfp*, and *ceh-26::gfp* transgenes are *adIs1240, mnIs17*, and *chIs1200,* respectively. Strains bearing *adIs1240* might have *lin-15(n765)* mutation in the background.

[c] Number of animals scored.

[d] These two strains also contain the transgene *mnIs17,* but *osm-6::gfp* expression was not scored.

[e] A weak hook induction in P(1-2).p is observed in this strain (6/138), which is probably due to interaction of activated LIN-12 signaling with the *adIs1240* transgene. Similar P(1-2).p hook formation is still observed after removal of *mnIs17*or *lin-15(n765)* from the background (data not shown).

[f] Animals were inspected at late L4 stage for *osm-6::gfp* expression in HOA and HOB. Hook invaginations, instead of hooks, were scored in those males.

[g] Data are cited from Yu et al. (2003).

[h] Often only one *osm-6::gfp*-expressing cell instead of a pair of hook neurons was observed.

Table 2. Ectopic HCG induction in *pry-1* mutants

Genotype[a]		HCG induction[b] (%)				avg. hook-like structure[c]	avg. marker expression	n[d]
signaling	markers	P(1-2).p	P(3-6).p	P(7-8).p	P(9-11).p			
+	none	0	0	0	100	1	NA	many
pry-1	"	0	74.1	30.9	43.2	2.04	NA	81
+	*eat-4::gfp* (1°)	0	0	0	100	1	1	117
pry-1	"	0	94.9	38.5	5.1	2.23	2.03	39
+	*ceh-26::gfp* (2°)	0	0	0	100	1	1	>2000[e]
pry-1	"	0	83.3	16.7	16.7	1.93	0.23	30
bar-1	"	0	0	0	86[f]	0.80	0.69	71
pry-1; bar-1	"	0	0	0	93[f]	0.96	0.93	147
+	*osm-6::gfp* (2°)	0	0	0	100	1	1	>200[e]
pry-1	"	0	92.6	37.0	22.2	1.81	0.33	27
lin-12(gf)/lin-12(lf)	"	0	0	0	100	2.3	2.3	69
pry-1; lin-12(gf)/lin-12(lf)	"	25	100	21.4	3.6	2.89	1.36	28

[a] Alleles used are: *pry-1(mu38)*, *lin-12(n137)* as *lin-12(gf)*, and *lin-12(n676n909)* as *lin-12(lf)*. Integrated array are *adIs1240(eat-4::gfp)*, *chIs1200(ceh-26::gfp)*, and *mnIs17(osm-6::gfp)*. All strains contain *him-5(e1490)* in the background.

[b] Percentage of animals in which Pn.p subgroups adopt HCG-like fates, determined by the presence of hook-like invagination or ventral protrusion, and/or ectopic lineage marker expression.

[c] Including hook-like invagination or ventral protrusion.

[d] Number of animals scored.

[e] Data are from a genetic screen described in Yu et al. (2003).

[f] Only the 2° fate is scored. Actual HCG induction including both the 1°and 2° is probably higher.

NA, not available.

Table 3. Hox genes are required for HCG fate transformation induced by Wnt signaling

genotype[a]			HCG induction[b] (%)				avg. hook-like structure[c]	avg. marker expression	n[d]
Wnt signaling	**Hox**	**markers**	**P(1-2).p**	**P(3-6).p**	**P(7-8).p**	**P(9-11).p**			
+	+	*eat-4::gfp* (1°)	0	0	0	100	1	1	117
pry-1	+	"	0	94.9	38.5	5.1	2.23	2.03	39
+	*mab-5*	"	0	0	0	0	0	0	84
pry-1	*mab-5*	"	0	0	0	0	0	0	83
+	+	none	0	0	0	100	1	NA	many
pry-1	+	"	0	74.1	30.9	43.2	2.04	NA	81
+	*lin-39*	"	0	0	0	100	1	NA	80
pry-1	*lin-39*	"	0	14	39.5	14	0.79	NA	43
+	*lin-39*	*eat-4::gfp* (1°)	0	0	0	100	1	1	63
pry-1	*lin-39*	"	0	4.2	56.3	25	0.83	0.88	48

[a] Alleles used are: *pry-1(mu38), mab-5(e1239), lin-39(n1760).* Both Hox mutant alleles are probably null. All strains contain *him-5(e1490)* in the background. The *eat-4::gfp* transgene is *adIs1240*.

[b] Some of the data in this table are present in Table 2.

[c] Including hook-like invaginations or ventral protrusions.

[d] Number of animals scored.

NA, not available.

Table 4. Ectopic HCG induction by increased MAB-5 activity and activated LIN-12 or EGF signaling

genotype[a]	markers	HCG induction (%)				avg. hook-like structure	avg. marker expression	n[b]
		P(1-2).p	P(3-6).p	P(7-8).p	P(9-11).p			
+	*osm-6::gfp* (2°)	0	0	0	100	1	1	>200[c]
mab-5(gf)	"	0	0	0	100	1	1	70
lin-12(gf)/lin-12(lf)	"	0	0	0	100	2.3	2.3	70
mab-5(gf)/lin-12(gf)	"	54.1	39.2	6.8	100	3.66	3.32	74
mab-5(gf)	none	0	0	0	100	1	NA	70
lin-15	"	0	0	0	100	1.47	NA	68
mab-5(gf); lin-15		54.7	11.3	0	84.9	2.09	NA	53
lin-15	*osm-6::gfp* (2°)	0	0	0	100	1.48	1	42
mab-5(gf);lin-15	"	87.1	19.4	9.7	93.5	2.97	0.74	31
lin-22	*osm-6::gfp* (2°)	0	0	0	100	1	1	82
lin-12(gf)/lin-12(lf); lin-22	"	5.5	5.5	27.3	100	2.64	2.36	55

[a] Alleles used are: *mab-5(e1751*), a gain-of-function allele; *lin-12(n137)* as *lin-12(gf)*, a gain-of-function allele; *lin-12(n676n909)* as *lin-12(lf)*, a loss-of-function allele; *lin-15(e1763*), a severe reduction-of-function allele; *lin-22(n372)*, a reduction-of-function allele. The *osm-6::gfp* transgene is *mnIs17*.

[b] Number of animals scored.

[c] Data from Yu et al. (2003).

Figure 1. Development of the male hook sensillum competence group. (A) Cell division patterns of P(9-11).p, adapted from Sulston et al. (1977). so, socket cell; sh, sheath cell. (B-D) Nomarski photomicrographs of wild-type male tail. The hook invagination or the hook is indicated by arrowhead. Positions of HOA, HOB, and PVV are indicated by arrows. (B, D) Late L4. (C) Adult. The hook structure (arrowhead) has yellowish autofluorescence if viewed under UV illumination (not shown). (E) Fluorescence image of the same L4 male tail as in (D) with *eat-4::gfp* expression. The GFP-positive cell is PVV. The exposure time used for this picture cannot show the processes of PVV. Left lateral views (anterior left, ventral down). Scale bar equals 20 μm.

Figure 1

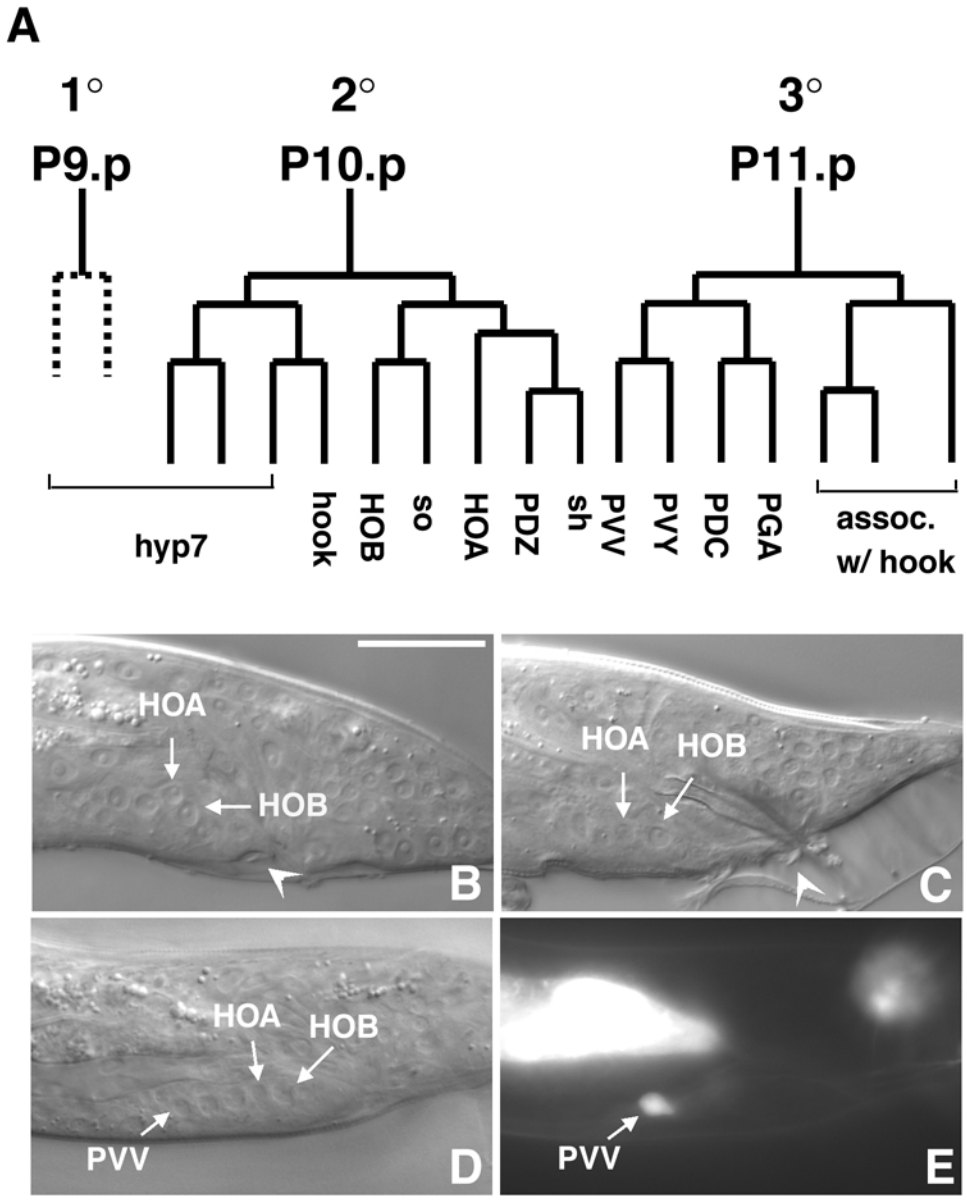

A
1°
2°
3°
P9.p
P10.p
P11.p
hyp7
hook
HOB
so
HOA
PDZ
sh
PVV
PVY
PDC
PGA
assoc.
w/ hook
HOA
HOB
B
HOA
HOB
C
HOA
HOB
PVV
D
PVV
E

Figure 2. *lin-17* expression in male P(9-11).p. Nomarski (A) and fluorescence (B) images of an early L3 male larva expressing a GFP reporter construct fused with *lin-17* promoter. GPF expression in P10.p is barely detectable. Nomarski (C) and fluorescence (D) images of a mid-L3 male larva after the first round of P(10-11).p divisions. P9.p in this animal is located more posteriorly than usual. P11.pa and P11.pp (anterior is to the left) have brighter *lin-17::gfp* expression than the two P10.p progeny. Scale bar, 20 μm. Left lateral views.

Figure 2

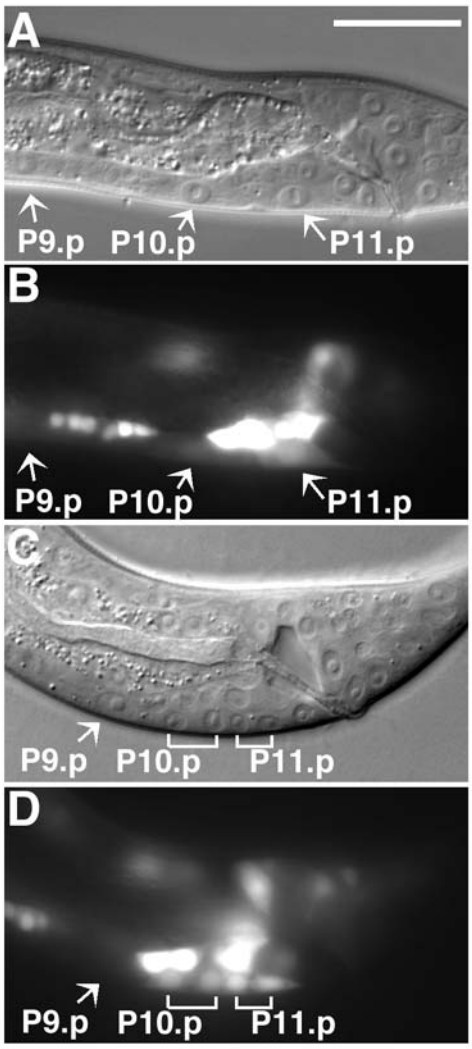


Figure 3. Dynamic expression of *bar-1::gfp* in male P11.p. The P11.p nucleus is labeled by a big arrow; other cells are indicated by small arrows. Nomarski (A) and fluorescence (B) images of an L1 male larva. P12 just divided. Faint *bar-1::gfp* expression is seen in both P12.a and P12.p, but not in undivided P11 (big cell, going to divide). Nomarski (C) and fluorescence (D) images of a late-L1 male larva. P12.p has divided. P12.pp underwent apoptosis and its corpse is indicated by a big arrowhead. Faint expression of *bar-1::gfp* is seen in P11.p. Nomarski (E) and fluorescence (F) images of a mid-L2 male larva. Bright cytoplasmic punctuate GFP staining (indicated by small arrowheads) and faint nuclear GFP expression (big arrow) in P11.p. Nomarski (G) and fluorescence (H) images of an early L3 male larva. P11.p has predominant nuclear expression of *bar-1::gfp* (big arrow) and fewer cytoplasmic GFP granules (small arrowheads). Nomarski (I) and fluorescence (J) images of a mid-L3 male larva right before the first round of P(10-11).p divisions. Exclusive nuclear *bar-1::gfp* expression in P11.p. In this panel, picture (D) and (J) used a longer exposure time. Scale bar, 20 μm. Left lateral views.

Figure 3

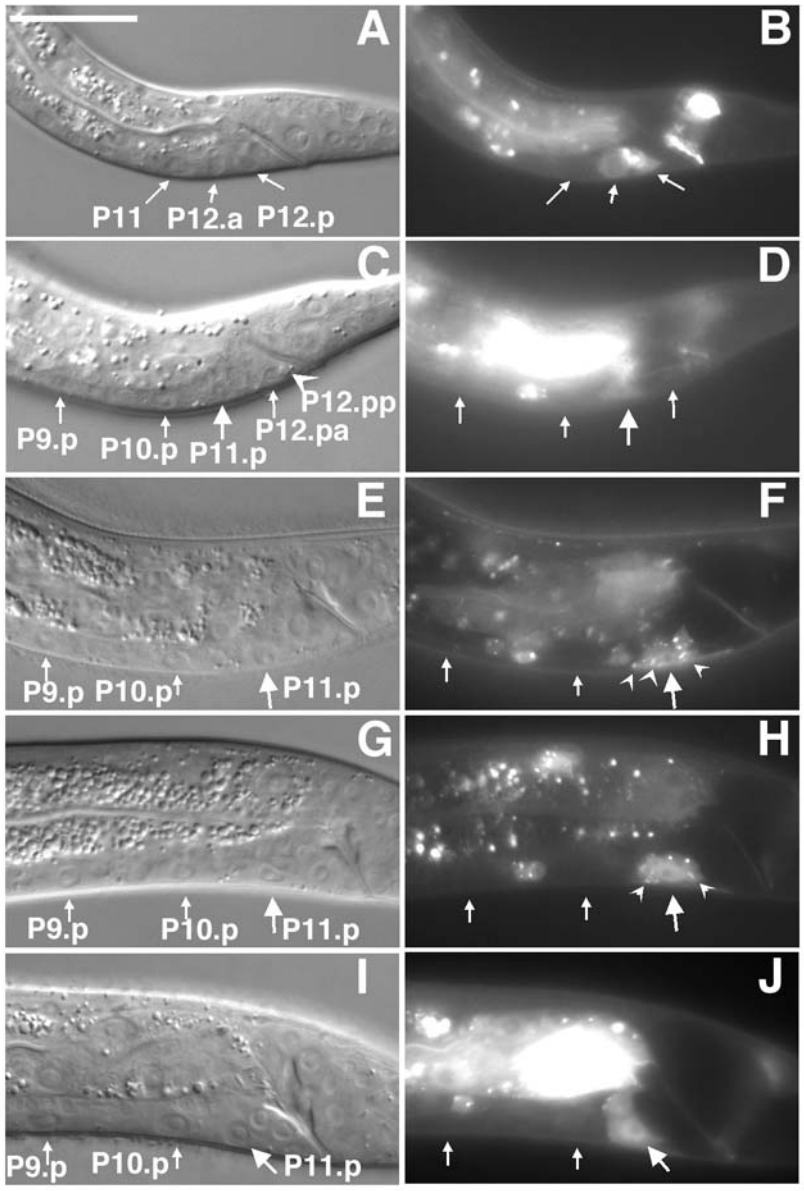

A
P11
P12.a
P12.p
B
C
P9.p
P10.p
P11.p
P12.pa
P12.pp
D
E
P9.p
P10.p
P11.p
F
G
P9.p
P10.p
P11.p
H
I
P9.p
P10.p
P11.p
J

Figure 4. Formation of ectopic HCG fates in *pry-1* mutant males. (A) Late L4. Two ventral invaginations with anchor-like shape inside (arrowheads) in the central region of a *pry-1(mu38)* male. (B) Overlay of Nomarski and fluorescence images of an adult *pry-1(mu38)* male with two ventral protrusions (arrowheads) in the middle body. The autofluorescence can be seen at the tip of the protrusions. Nomarski (C) and fluorescence (D) images of an adult *pry-1(mu38)* male with an autofluorescent ventral protrusion (arrowhead) and an associated ectopic HOB hook neuron as indicated by *ceh-26::gfp* expression (arrow). (E-F) Correlation of ectopic *eat-4::gfp*-expression neurons (arrows) with ventral protrusions (arrowheads) in a *pry-1(mu38)* male. Scale bar, 20 μm. Left lateral views.

Figure 4

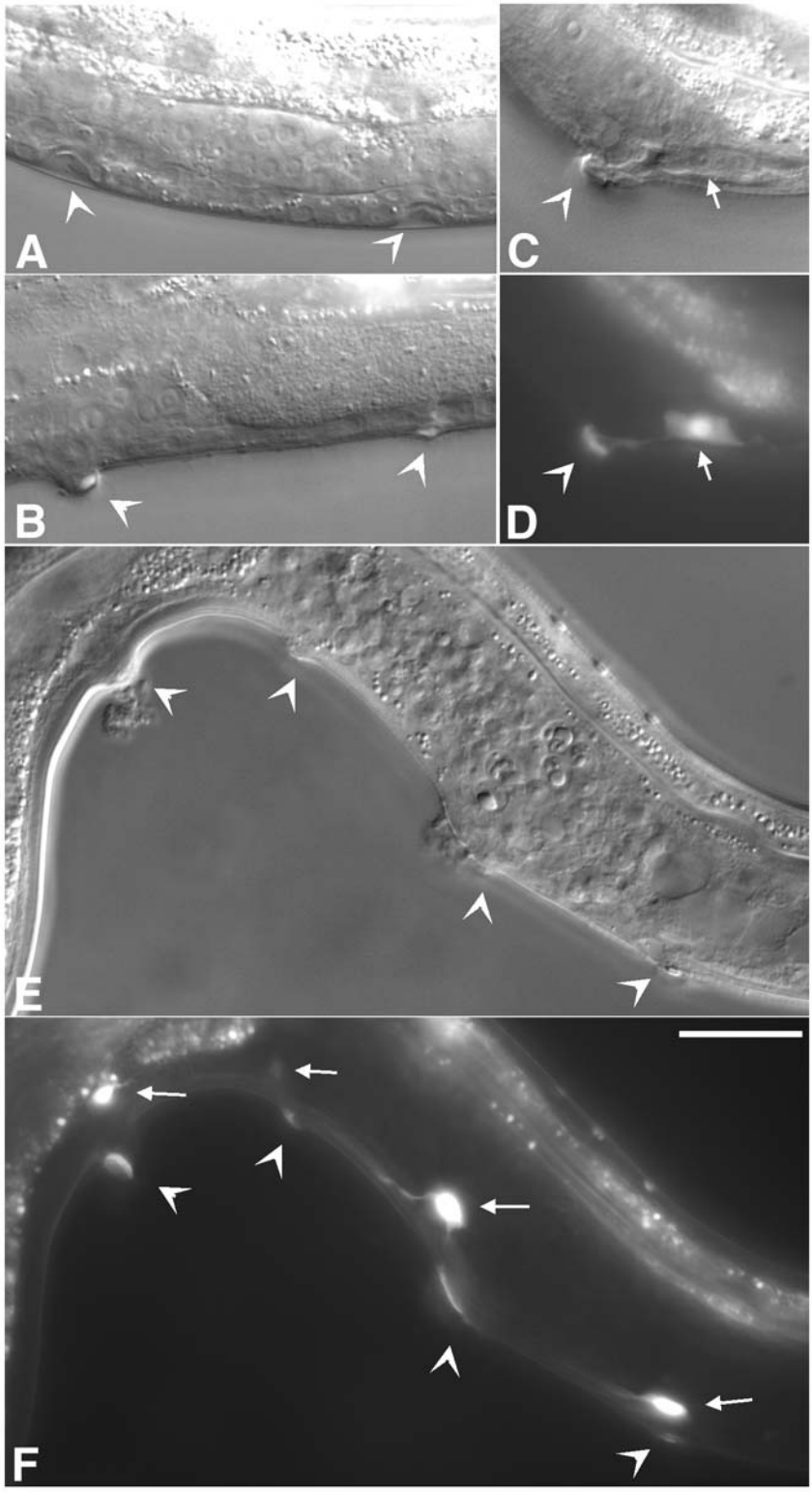


Figure 5. Ectopic 2° fate formation in anterior Pn.p cells by activated Wnt and LIN-12 signaling. Nomarski (A) and fluorescence (B) images of an L4 *pry-1; lin-12(gf)/lin-12(null)* male with three hook invaginations (arrowheads) and three pairs of hook neurons (arrows) among the middle Pn.p cells. Scale bar, 20 μm. Left lateral views.

Figure 5

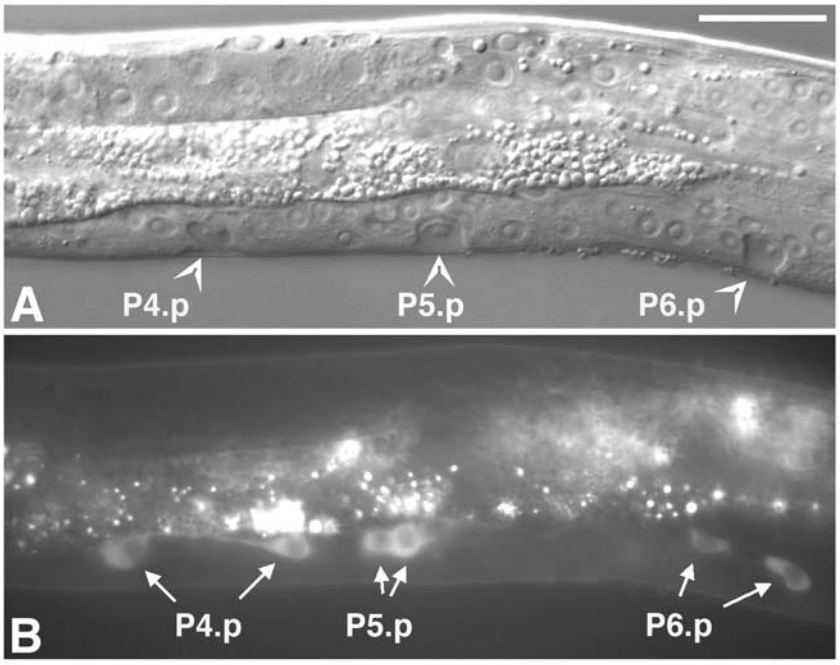


Figure 6. Collaborative consequence of constitutive MAB-5 activity and Notch or EGF signaling. (A-B) 2°-like HCG fate transformation in the central Pn.p cells by increased MAB-5 activity and activated LIN-12 signaling. Nomarski (A) and fluorescence (B) images of an L4 *mab-5(e1751gf)/lin-12(gf)* male with hook invaginations at P5.p and P6.p (arrowheads). The P5.p invagination was associated with a pair of hook neurons (arrows). (C-E) Ectopic hook formation in P(1-2).p by extra MAB-5 activity and EGF signaling. (C) An L4 *mab-5(e1751gf); lin-15(e1753)* male with two hook-like invaginations at P(1-2).p. Nomarski (A) and fluorescence (B) images of a P1.p hook in an adult *mab-5(e1751gf); lin-15(e1753)* male. Scale bar, 20 μm. Left lateral views.

Figure 6

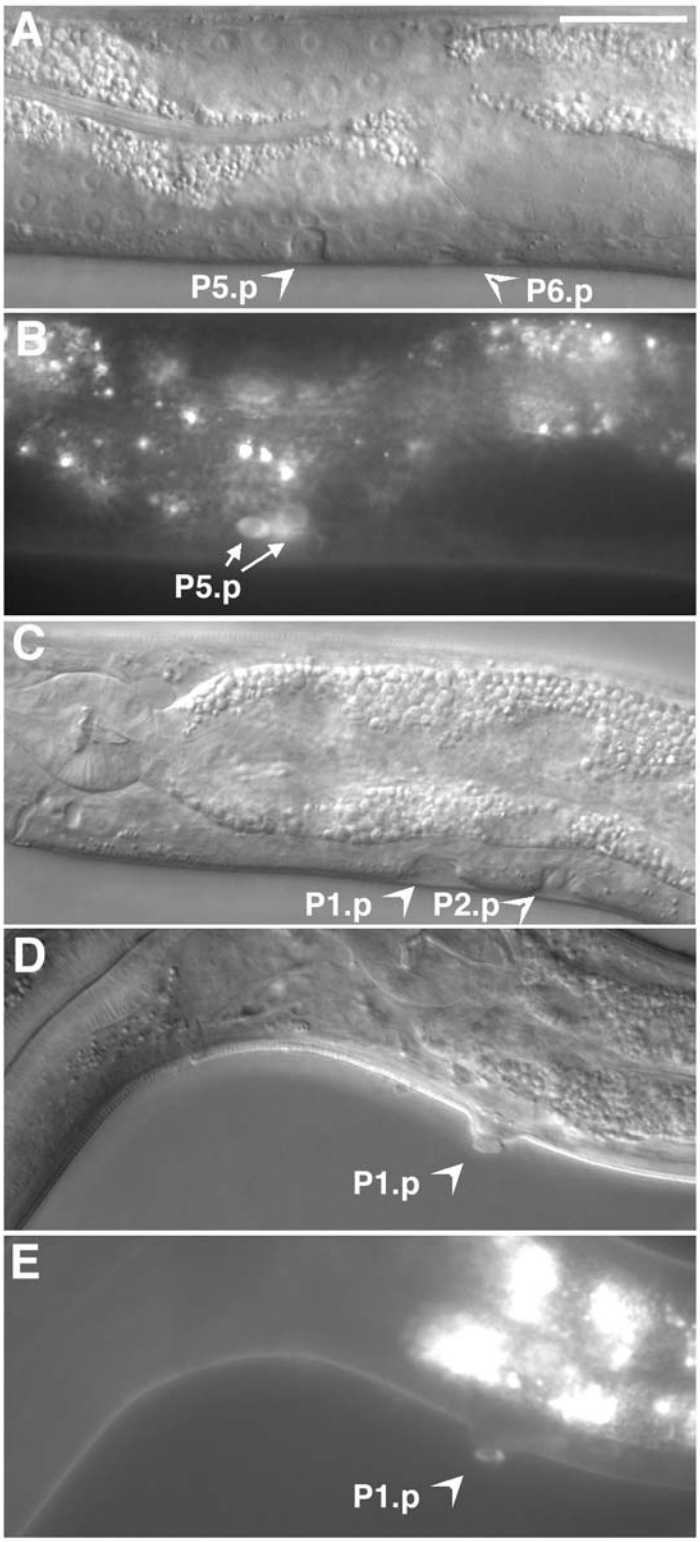


Figure 7. 2° HCG fate formation in male Pn.p cells by interaction between activated LIN-12 signaling and a *lin-22* mutation. (A) An L4 male with two hook-like invaginations at P1.p and P2.p (arrowheads). Nomarski (B) and fluorescence (C) images of an L4 *lin-12(gf)/lin-12(null); lin-22(n372)* male. In addition to a P10.p wild-type hook sensillum, a P9.p hook sensillum is seen in *lin-12(gf)/lin-12(null)* males. P(7-8).p are also induced to form hook invaginations in this *lin-12(gf)/lin-12(null); lin-22(n372)*male . The P7.p hook invagination is associated with a pair of hook neuron, therefore a complete 2° HCG fate transformation. The 2° fate formation of P8.p is partial as only hook invagination is seen. One cell of the P9.p pair is located in a slightly right focal plane therefore is not shown in the picture. Scale bar, 20 μm. Left lateral views.

Figure 7

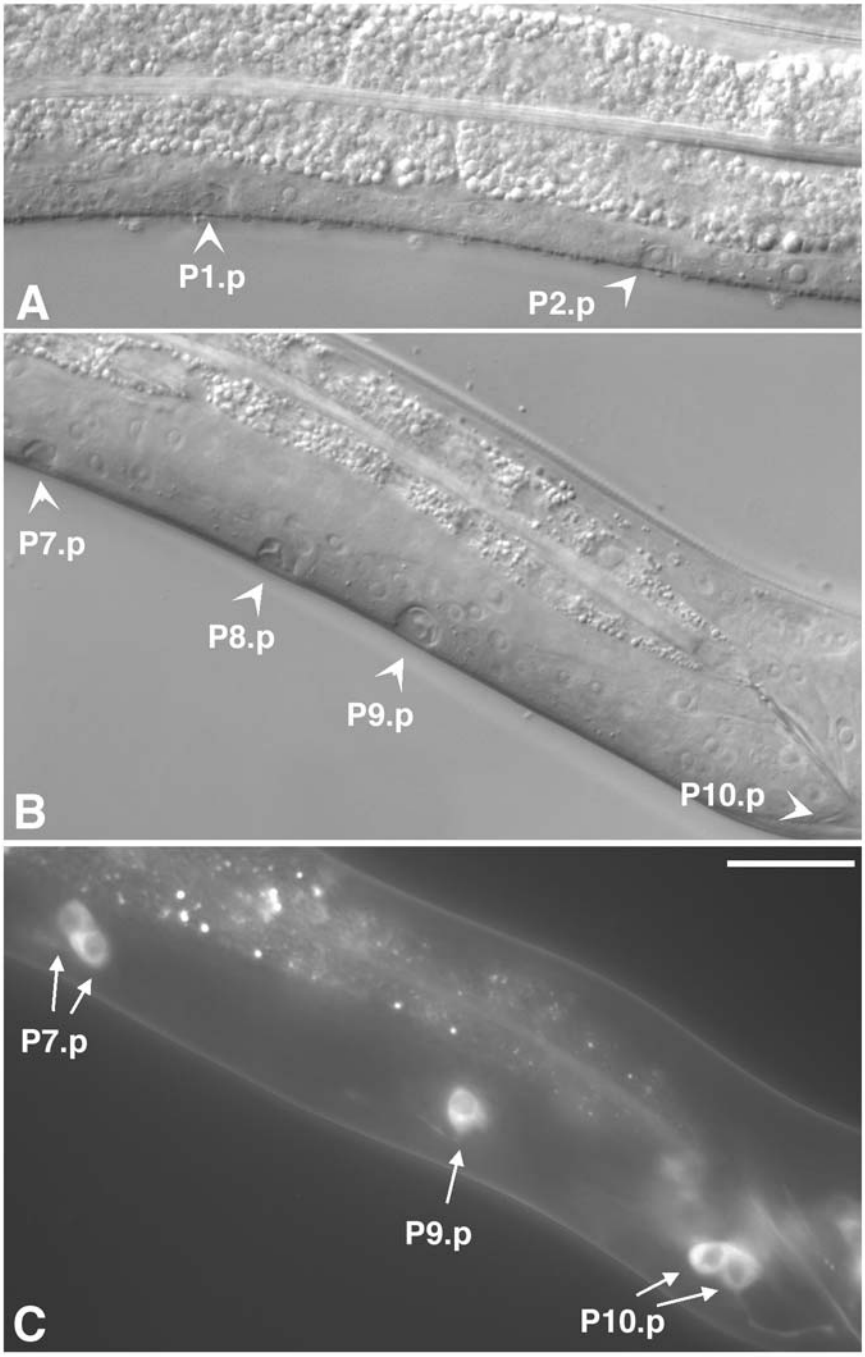
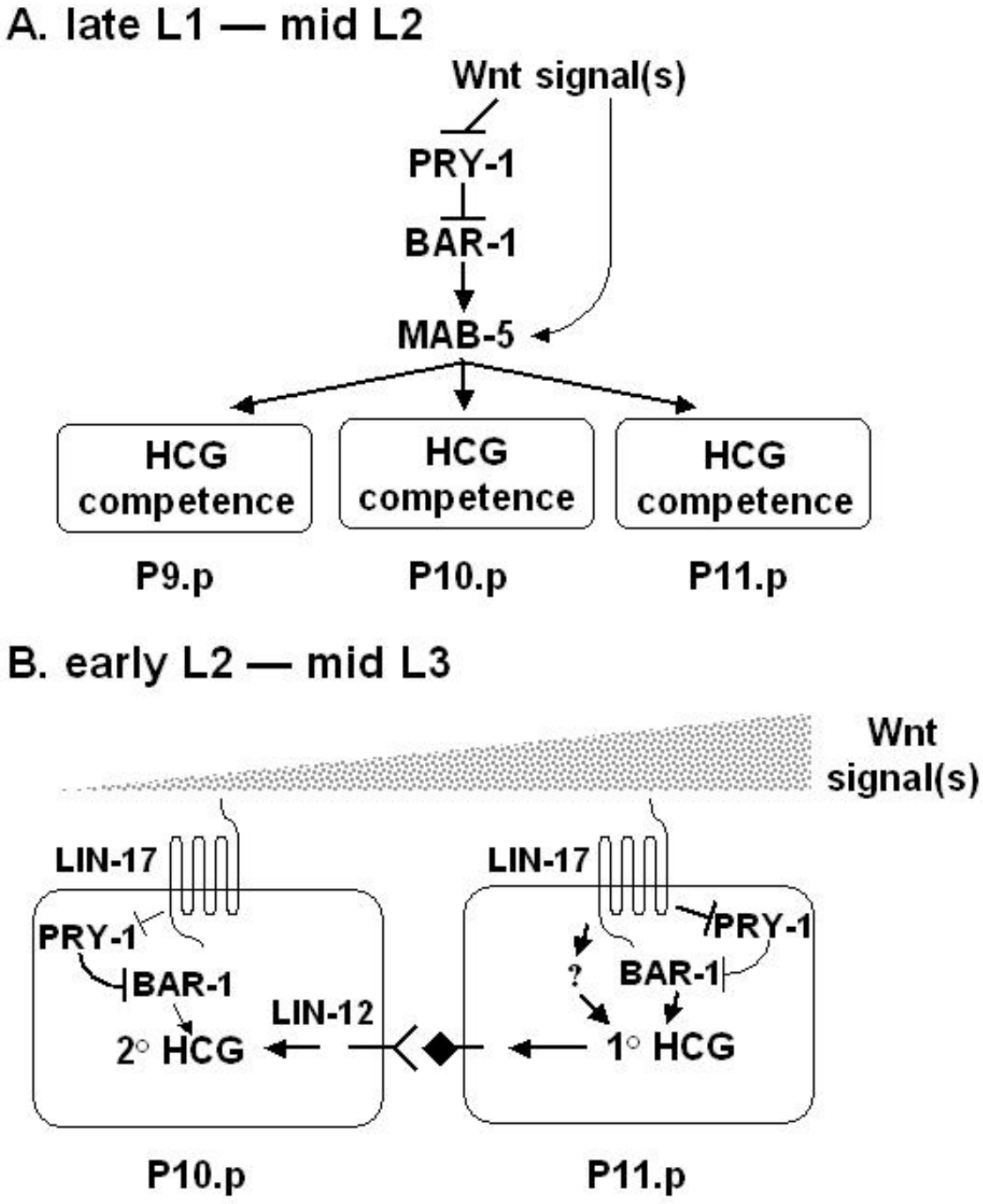


Figure 8. Model of HCG fate specification. P(9-11).p remain unfused in the late L1 stage by LIN-17-independent MAB-5 activity. From the late L1 to middle L2 stage, a Wnt signal or signals graded from posterior tail region regulates *mab-5* activity to establish HCG competence in P(9-11).p cells. The P9.p cell, which receives the least Wnt signal(s), has the 3° fate and usually fuses with hyp7 during the late L2 stage. In response to a high level of Wnt signal(s), LIN-17 receptor on the cell surface of P11.p activates downstream pathway to specify the 1° fate, which produces ligands for LIN-12. In P10.p, activated LIN-12 signaling by adjacent 1° P11.p cell acts with a weak Wnt signal to promote the 2° HCG fate.

Figure 8


A. late L1 — mid L2
Wnt signal(s)
PRY-1
BAR-1
MAB-5
HCG competence
HCG competence
HCG competence
P9.p
P10.p
P11.p
B. early L2 — mid L3
Wnt signal(s)
LIN-17
PRY-1
BAR-1
2° HCG
LIN-12
LIN-17
PRY-1
?
BAR-1
1° HCG
P10.p
P11.p

APPENDIX

Analysis of other factors associated with the Wnt pathway

Expression of Wnt homologs in the male tail

To identify the possible inductive signal for HCG patterning, the expression patterns of three Wnt proteins were examined. Five Wnt homologs, *lin-44*, *egl-20*, *mom-2*, *cwn-1* and *cwn-2*, are found in the *C. elegans* genome (Korswagen, 2002). It has been reported that *egl-20* is expressed in the anal depressor muscle, and in the male specific blast cells P9/10, K, F, U, and B in the tail (Whangbo and Kenyon, 1999). I examined *cwn-1* and *cwn-2* expression in animals containing the extrachromosomal array *syEx556 (cwn-1::gfp)* or *cwn-2::gfp* arrays *syEx631* and *syEx566* made by Takao Inoue. Two cells on top of P11.p, likely DP6 and DA8, express *cwn-1::gfp*. Other *cwn-1* expression is seen in the diagonal muscles, the anal depressor muscle, and cells in the ventral cord. From L4 through adulthood, the hook neuron HOB expresses *cwn-1::gfp* as well. *cwn-2::gfp* is expressed in rectal gland cells (the expression is not sex-specific and often only in the dorsal one), a tiny cell just posterior to the anus, which could be the embryonic neuron LVAL, and another cell which probably is the blast cell K. Some *mom-2::gfp* expression is present in the tail region but has not been carefully examined. In hermaphrodites, *lin-44* is expressed in the tail hypodermis (Herman et al., 1995).

No hook sensillum defect was observed in *egl-20(n585)* mutants. The *mom-2(or42)* mutation causes maternal embryonic lethality. The viable *mom-2(or42)* males descended from heterozygous mothers have normal tail morphology in general and can mate to produce cross-progeny.

Expression and mutant phenotype of *lin-18* RTK in male P(9-11).p lineages

lin-18 encodes a receptor tyrosine kinase with a WIF domain at the extracellular region and acts complementary with the *lin-17* Wnt-like receptor to determine cell polarity during the 2° fate execution of P5.p and P7.p VPCs (Inoue et al., 2004). *lin-18::gfp* expression is almost undetectable in P(9-11).p before cell divisions (expression in P10.p is higher than that in P11.p if there is any). From the two-cell stage to the completion of cell divisions, both P10.p and P11.p progeny express *lin-18::gfp,* and the expression in the 2° P10.p lineage is always brighter. This pattern is opposite to that of *lin-17::gfp* in P(10-11).p lineages. A *lin-18(e620)* mutation has little effect on formation of the hook sensillum. Only 1 out of 25 *lin-18(e620)* males had a misshapen hook with normal expression of a HOB marker, *ceh-26::gfp*. In another *lin-18(e620)* strain containing no *ceh-26::gfp* transgene, 3/25 males had a misshapen hook.

Overexpression of FrzB-like gene *Y73B6BL.21* using a heat shock promoter

Secreted Frizzled-related proteins (FRP/FrzB) are Wnt antagonists, containing the cysteine rich domain (CRD) of Frizzled but lacking a transmembrane domain (Jones and Jomary, 2002). FrzB proteins can bind to Wnt proteins to modulate Wnt signaling. The *C. elegans* genome has only one FrzB-like gene, *Y73B6BL.21* (Korswagen, 2002). The predicted *Y73B6BL.21* open reading frame is 783 bp, and consists of 5 small exons scattered in a 5161 bp genomic region by large introns (WS 128). To determine whether *Y73B6BL.21*has an effect on Wnt signaling, I used a heat shock promoter to overexpress *Y73B6BL.21*. Specifically, a ~6kb genomic fragment containing *Y73B6BL.21*was PCR

amplified and inserted downstream of the *hsp-16.2* promoter using the Kpn I and Sac I sites of the vector pPD49.83. The *hsp::Y73B6BL.21*construct was injected into the strain *unc-119(ed4); him-5(e1490)* at 50 ng/μl using *unc-119(+)* plasmid pDP#MM016B (50 ng/μl) as a co-injection marker. Eleven independent stable lines (three high-transmitting lines and eight low-transmitting lines) were obtained based on rescue of the Unc phenotype of *unc-119*. Plates with animals at various stages were subjected to a 30-minute heat shock treatment at 33°C for all eleven lines, and then were monitored over the next couple of days for possible phenotypes. Several lines were tested again in another separate trial. None of these transgenic lines exhibited any obvious abnormality. Slightly Pvul (protruding vulva) hermaphrodites were observed occasionally. It is possible that the activity of *Y73B6BL.21*is tightly regulated and that its large introns carry regulatory elements that might keep the transgene silent. Or, *Y73B6BL.21* might not have any obvious function in *C. elegans*. Decreasing *Y73B6BL.21* transcript level by RNAi feeding with a construct including most of the *Y73B6BL.21* cDNA had no effect (T. Inoue, unpublished). Another possibility is that the transgenic lines did not really incorporate significant levels of the *hsp::Y73B6BL.21* transgene.

Mutant phenotypes of different components of the Wnt pathway— *hsp-16.1::ΔNT-bar-1, wrm-1, pop-1, mig-2, egl-8 (PLC-β)*

To test if *bar-1* overexpression mimics the *pry-1* phenotype, to induce ectopic HCG fates, I used *huIs1*, a transgene that contains a N-terminal truncated *bar-1* gene driven by heat shock promoter *hsp-16.1 (hsp-16.1::ΔNT-bar-1)* to overexpress *bar-1* (Gleason et al., 2002). Deletion of the N-terminal region of β-Catenin removes GSK3β sites and thus

stabilizes BAR-1 protein (Gleason et al., 2002). Animals were heat shocked at five different developmental stages, late L1, mid-L2, late L2, mid-L3, and late L3, which were estimated by number of hours after one hour egg-lay at 20°C. The heat shock treatment was performed at 33°C for 30 minutes. About 50% of hermaphrodites heat shocked during mid- to late L2 had a Muv phenotype, some of the other 50% were Pvul. No obvious effect was seen for hermaphrodites heat shocked at the L1 stage. Heat shock during L3 caused a Pvul phenotype in a few hermaphrodites. Males heat-shocked in L1 and L2 were growth arrested, small, and the tail region was severely deformed. Most tail structures in these males were gone, and the HOB hook neuron, indicated by *ceh-26::gfp*, or rudimentary hook-like protrusion was only occasionally observed. Heat shock at the L3 stage caused less severe tail defects, including fused rays, crumple spicules, sometime misshapen hook and/or abnormal HOB expression of *ceh-26::gfp*. The detrimental effect on males by *bar-1* overactivation was manifest even when the heat shock time was reduced to 15 minutes. However, heat shock at a lower temperature, 30°C, for 30 minutes barely affected males. Therefore, the heat shock condition tested so far has not been optimized for examining *bar-1* function during male HCG induction.

wrm-1 is another *C. elegans* β–catenin gene that plays a role in embryonic development. Zygotic RNAi of *wrm-1* produces male tail defects (Herman, 2001). *ne1982* is a *ts* allele (a gift from Craig Mello, University of Massachusetts) that disrupts *wrm-1* function at the non-permissive temperature of 25°C. I examined the 1° fate marker *eat-4::gfp* expression and hook morphology for *wrm-1(ne1982)* males growing at 25°C. 13/13 males had PVV expression of *eat-4::gfp*, but 6/13 had no hook structure. Among six hookless animals,

five had an dot of autofluorescent cuticle just anterior to the cloaca, resembling a feature generated by the three posterior P11.p progeny. Therefore, the 1° fate appears normal in a *wrm-1* mutant but formation of a 2° hook is affected. Similar results were also seen in a *wrm-1(ne1982); mnIs17(osm-6::gfp) him-5(e1490)* strain: 19/67 males had a wild-type hook, 16/67 had a misshapen hook, 26/67 had no hook but showed the 1° autofluorescent cuticle, 5/67 had no hook and unknown 1° fate, 1/67 might have two rudimentary hooks probably due to the 2° fate adoption in both P10.p and P11.p after P12-to-P11 transformation. In general, the HCG defect caused by a *wrm-1* mutation is similar to the *bar-1* mutant phenotype, supporting the hypothesis that these two β–catenins work in concert during HCG patterning.

pop-1 encodes a TCF homolog that acts with β–catenin in the Wnt pathway to activate transcription (Herman, 2001). I examined *pop-1(zu189)* homozygous males that were obtained from a heterozygous mother and found no obvious defect in the hook sensillum. Overexpression of a dominant negative form of *pop-1* by the heat shock promoter using *huIs4* transgene (Herman, 2001) rendered a mild defect in the hook sensillum: after heat-shock treatment for 30 minutes at 33°C during L2-L3, 2/18 males had a normal hook, 1/18 males had a misshapen hook, 13/18 males had an anterior hook, and 2/18 males had no hook. This phenotype is no more severe than the *bar-1* mutant phenotype, indicating that other Wnt downstream pathways might be involved in HCG patterning.

mig-2 encodes a Rac-like GTPase, a member of the Rho family (Zipkin et al., 1997), and thus could be a downstream component in the non-canonical Wnt pathway. I analyzed

male tail morphology in two *mig-2* mutants, *gm103* and *rh-17*. The *mig-2* mutants examined contained crumpled spicules but the majority had a normal hook sensillum (38/49 for *gm103* and 39/45 for *rh17*). A few *mig-2* mutants displayed defects in the hook sensillum: 4/49 of *mig-2(gm103)* and 1/45 of *mig-2(rh17)* males had an anterior hook (HOB might have been located anteriorly too), 5/49 of *mig-2(gm103)* and 3/45 of *mig-2(rh17)* animals lost either the hook structure or the HOB hook neuron, and 1/49 of *mig-2(gm103)* and 2/45 of *mig-2(rh17)* males lacked both the hook structure and HOB.

egl-8 is the only PLC-β found in the *C. elegans* genome (Lackner et al., 1999; Miller et al., 1999) and might mediate the Wnt/Ca++ pathway. However, no obvious defect in HCG lineages was observed in *egl-8(n488)* mutants.

Additional analysis of the Hox gene *mab-5*

mab-5 expression in male P(9-11).p

I examined *mab-5* expression in male P(9-11).p using an integrated *mab-5::gfp* transgene *muIs16* (Hunter et al., 1999). In general, wild-type animals have bright *mab-5::gfp* expression in many cells in the posterior part of the body (several of which are ray neurons and sex muscles). Only about half of the examined males had detectable *mab-5::gfp* expression in P(9-11).p, often just in P11.p (13/25), sometimes in P10.p (4/25) and P9.p (2/25), as well as P12.pa (4/25). This pattern is different from the previously described *mab-5* expression pattern in Pn.p cells. The *mab-5::gfp* construct uses the same 15kb 5' upstream region as the published *mab-5::lacZ* construct (Hunter et al., 1999). It is unclear whether the difference in expression is due to some specific feature of the GFP

protein. Alternatively, the 15kb upstream region actually contains some regulatory elements for another Hox gene *egl-5* therefore the observed GFP reflects both *mab-5* and *egl-5* expression. In *pry-1(mu38)* mutants, overall *"mab-5"::gfp* expression was increased and extended to anterior regions. Expression in male posterior Pn.p cells was also increased but P11.p still had the most detectable GFP expression within the group: GFP in 8/19 P9.p, 10/19 P10.p, 17/19 P11.p, and 10/19 P12.pa. No obvious difference was seen for expression of *muIs16* transgene in *lin-22(n372)* mutants, and posterior Pn.p cells with GFP expression were 1/14 P9.p, 2/14 P10.p, 8/14 P11.p, and 5/14 P12.pa.

Heat shock-induced MAB-5 overexpression during the middle L2 to early L3 stage has a negative effect on hook formation

To test if an ectopic hook is induced by overexpression of *mab-5*, I took advantage of a transgenic line *muIs9* that expresses *mab-5* under the control of an *hsp-16* promoter (Salser et al., 1993). A time window from the mid-L2 to early L3 stage was selected for heat shock treatment, as it is the approximate determination time for the HCG (Herman, 1991). Extensive overexpression of *mab-5* during the mid-L2 to early L3 stage by heat shock led to a high percentage of hook abnormalities in adult males, including no hook, misshapen hook, or anterior hook (Table 1). However, a second hook was not observed. Based on the *ceh-26::gfp* expression, HOB was less affected by *hs-mab-5*. Occasionally, a second cell expressed *ceh-26::gfp* after the heat shock treatment. In *mab-5(e1751gf); lin-15(e1763)* and *mab-5(e1751)/lin-12(gf)* strains, we also noticed a third *osm-6::gfp*-expressing cell in addition to HOA and HOB in a few males. This cell is usually located posteriorly next to HOB. It is not clear whether this is a HOB-like fate transformation

within the 2° lineage due to excessive MAB-5 activity. Formation of ectopic hook in *lin-12(gf)* males was also blocked by *hs-mab-5* (Table 2). The animals without the *hs-mab-5* transgene were wild type after the same heat shock treatment. Insufficient ectopic hook induction by heat shock-induced MAB-5 overexpression is consistent with other findings (see the Chapter 2) that *mab-5* plays a permissive role in HCG fate specification.

It has been shown that *mab-5* expression reaches peak levels about 1.5 hours after the start of the heat-shock treatment, becomes weak by 6 hours, and lasts until 9 hours. Therefore, a heat-shock at the late L2/early L3 stage could result in extra *mab-5* activity in the late L3 to the early L4 stage. The observed disruption of hook formation by overexpression of *hs-mab-5* implicates that too much *mab-5* activity at the later stage might have a negative effect on the 2° fate execution and/or hook sensillum differentiation, although *mab-5* function is required at an earlier time for HCG patterning. It is probably necessary to examine expression of the 1° fate marker, *eat-4::gfp,* after heat shock induced *mab-5* overexpression as well. It will be interesting to know if there is any 1°/2° hybrid fates produced by excessive *mab-5* activity.

I tried using heat shock to induce *mab-5* expression in *mab-5(lf)* mutants during the L1 stage to see if blocking P(9-11).p fusion is sufficient to rescue the hookless defect. No hook formation was observed (several animals might have divided P11.p and P10.p). A few males had formed some V ray (5/32), indicating that heat shock induced *mab-5* expression at least functioned in some animals to partially rescue the ray defect.

Therefore, the lack of rescue of the hookless phenotype suggests that *mab-5* probably is required in L2-L3 for hook formation.

Timing of HCG fate determination

As HCG cells are all multipotent, a key issue is when these three cells are induced to their assigned fates. The time of determination of the three HCG fates has been investigated previously, but results from two different experiments left uncertainty. Fate replacements after cell killing reveal that the middle L2 stage (about twenty hours of postembryonic development) is the latest time point that an anterior cell is able to substitute a missing posterior cell fate within P(9-11).p group (Sulston and White, 1980; Herman, 1991). By contrast, suppression of the multi-hook phenotype of *lin-12(gf)* mutation by a temperature-sensitive mutation, *sup-17(n1260ts),* suggests that suppression can be effective as late as mid-L3 right before P(10-11).p divisions (Herman, 1991). Our analysis of *ajm-1::gfp* expression in male P(9-11).p is consistent with the earlier determinative time. AJM-1::GFP is localized to apical junctions of epithelial cells, marking cell-cell boundaries of unfused cells. A Pn.p cell, if unfused, has a ring of *ajm-1::gfp* on the ventral side (Sharma-Kishore et al., 1999; Gupta et al., 2003). Besides P(9-11).p, four middle Pn.p cells, male P(3-6).p, also remain unfused during L1 fusion because of *lin-39* activity. We observed *ajm-1::gfp* rings under nuclei of P(3-6).p and P(9-11).p up to the middle L2 stage. From the middle to late L2 stage, P(3-6).p cells and the majority of P9.p cells gradually lost *ajm-1::gfp* expression. A ring of *ajm-1::gfp* expression at P9.p was only seen in 2/12 early L3 males, and in both cases, P9.p was obviously posterior, which probably corresponds to the occasional situation in which

P9.p divides once. Therefore, both undivided P9.p and non-HCG-fated P(3-6).p cells fuse with the hyp7 epidermal syncytium during the middle to late L2 stage, the same temporal interval that *bar-1::gfp* expression exhibits dramatic change in P11.p (see the Chapter 2), suggesting a time window critical for HCG fate specification. There might be some further refining of the 1° and 2° HCG fates up to initiation of P10.p and P11.p cell divisions in the middle L3 stage.

Cell interactions involved in HCG patterning

An extension of cell ablation for identification of positional cues

The cell-killing performed by M. A. Herman (1991), J. E. Sulston and J. G. White (1980) is quite extensive. However, ablation of P12.p was never combined with other cells. P12.p or its progeny P12.pa is the next posterior neighbor of P11.p. It is possible that P12.p or P12.pa produces one of the positional cues. I ablated P12.p or P12.pa with B (or B.a and B.p), F, U, Y.a, and Y.p in either late L1 or early L2. One male (P12.pa ablated) with *osm-6::gfp* and three males (two P12.pa ablated, one P12.p ablated) with *ceh-26::gfp* survived to adulthood. All four animals have a lump of cells at the cloaca but no hook structure was seen. However, two out of three *ceh-26::gfp* males (one P12.pa and one P12.p) retain expression of the HOB-GFP marker, indicating that P(9-11).p lineage is probably not altered after ablation, although the hook morphogenesis might be affected. Animals with K.a, K.p, F, U, Y.a, Y.p, B.a, B.p, and P12.pa ablated were growth-arrested and thus were not examined for HCG patterning.

The 2° fate is intrinsic to the P10.p cell after its posterior migration

Expression of a 2° fate depends on the presence of a 1° fate cell (Sulston and White, 1980; Herman, 1991), indicating that lateral signaling is involved. To determine whether the dependence of a 2° fate on a 1° fate has a time limitation, I ablated P11.p at two different stages, before and after normal P10.p migration. I also killed P9.p to diminish the possible ambiguity caused by a P9.p 2° fate transformation. In terms of hook morphology and HOB neuronal identity, the 2° fate in an isolated P10.p is not altered by the disruption of 1° fate P11.p in the early L3 stage after P10.p has migrated (Table 3). However, the hook position was anterior in those animals. The debris from P11.p killing may block the hook migration. Or, cells from the P11.p lineage, e.g., the three posterior P11.p progeny, are required for hook migration during L4. Removal of anterior daughters of P11.p did not alter the hook position (Table 3).

The hook socket cell is required for formation of the hook structure

Within the 2° lineage, I found that ablation of the hook socket cell or its parent cell P10.ppa, but not the hook neurons, has a negative effect on hook structure (Table 3). Although the hook neurons send processes to connect the hook, which is probably important for their final functions in mating behavior, the presence of the hook neurons is not required for a normal hook structure. As to the HOA and HOB interactions, the results from the HOA ablation were ambiguous. 2/5 operated animals display a loss of *ceh-26::gfp* in presumptive HOB (Table 3). It is not clear if this implies a real influence on HOB neuronal identity by HOA, or it is simply because HOB was accidentally damaged during HOA killing. More ablations will help us to clarify this.

LIN-12 signaling in the 2° HCG fate

2° fate formation in *lin-12* mutants

Using the *osm-6::gfp* marker for the hook neurons, I demonstrated that activated LIN-12 signaling is sufficient for 2° fate execution. In *lin-12(gf)* mutants, each ectopic hook is accompanied by a pair of ectopic hook neuron(s), indicating a complete 2° fate transformation. Consistently, no PVV expression of *eat-4::gfp* was detected in animals with three hooks, suggesting that the 1° cell fate is no longer present.

lin-12(null) mutant males are hookless, due to the absence of 2° fate caused by deficient LIN-12 signaling (Greenwald et al., 1983). I found that PVV expression of *eat-4::gfp* is normal in 3/3 *lin-12(null)* males, suggesting that LIN-12 signaling only functions in 2° specification and does not affect the 1° fate.

Expression of *lip-1::gfp* responds to activation of LIN-12 signaling

lip-1 encodes a MAP kinase phosphatase and is regulated by LIN-12 signaling during 2° VPC induction (Berset et al., 2001). I examined *lip-1::gfp* using the *zhIs4* transgene in 10 males at the late L1/early L2 stage, 8 males in the middle to late L2 stage (B lineage at the 3-7 cell stage), 9 males in the late L2 to middle L3 (before P10 and P11.p divisions, B lineage at the 9-10 cell stage), and 6 males in the middle to late L3 stage (P10.p and P11.p divided). *lip-1::gfp* was first expressed uniformly in Pn.p cells. After the middle L2 stage, *lip-1::gfp* expression in P10.p was increased. Strong *lip-1::gfp* expression was observed in P10.p by the late L2/early L3 stage, while expression in P11.p was greatly decreased and became undetectable at the time of P11.p division. Interestingly,

expression of *lip-1::gfp* was also elevated in the posterior cell P12.pa, probably due to activation of LIN-12 signaling by the nearby 1° P11.p cell. P.9.p and other anterior Pn.p cells retained low *lip-1::gfp* expression.

Role of EGF signaling in HCG patterning

Incomplete 2° fate transformation in *lin-15(lf)* mutants

e1763 is a loss-of-function allele of *lin-15* (Ferguson and Horvitz, 1985). Mutations in *lin-15* affect P(9-11).p cell fates in two aspects. About half of *lin-15(e1763)* males had a P11-to-P12 transformation, which consequently confers to P10.p the 1° fate and P9.p the 2° fate, resulting a single hook mislocated anterior to the cloaca (P. W. Sternberg, unpublished). In those cases in which P11 is not affected, the *lin-15* mutation induces a 2°-like fate in P9.p, to generate a rudimentary hook in addition to the normal P10.p hook. Visualized by *ceh-26::gfp* as the hook neuron marker, although about 35% of animals had two hooks, no ectopic hook neuron was detected (n=55), indicating that the 2° fate transformation of P9.p induced by a *lin-15* mutation is not complete. A similar result was also obtained using the *osm-6::gfp* marker (see the Chapter 2). Overall, neither the hook structure nor hook neuron was detected in *lin-15* mutants (3/55) occasionally; in a few other cases (9/55), the hook neurons were absent in *lin-15(e1763)* males regardless if there is an ectopic hook, indicating a low frequency of deficient 2° fate induction. This observation also suggests that hook neurons are not necessary for forming the hook structure. *lin-15(e1763)* males are also Muv, making pseudovulvae at P(3-8).p. *egl-17::gfp* expression was observed at Muv bumps of *lin-15(e1763)* males but was not detected in P(9-11).p lineages. Both male Muv and ectopic hook phenotypes of *lin-15*

mutants were fully suppressed by *sy97*, a *let-23* severe reduction-of-function mutation, suggesting that the phenotype of *lin-15(lf)* males is due to excess *let-23* signaling. Interestingly, both two P11.p (caused by P12-P11 transformation—a phenotype of *let-23(97)*) and two P12.pa (caused by P11-P12 transformation—a phenotype of *lin-15(lf)*) were observed in *let-23(97); lin-15(e1763)* mutants, indicating a certain degree of mutual suppression.

Activated *let-23/let-60(ras)* signaling induces cell division in P9.p

To examine the effects of the *let-23/let-60* pathway, I varied RAS signaling at the L2 to L3 stage using two transgenic lines, *syEx286* (an *hsp-16-let-60 ras* active form (*da*)) and *syEx284* (a *ras* dominant negative form (*dn*)) made by M. Wang. The *ras dn* form showed no obvious effect and animals had a wild type hook (n=67). This is consistent with the previous finding that *let-23* mutation does not disrupt the 1° and 2° HCG fate induction (P. W. Sternberg, unpublished). After the heat shock treatment, *ras da* males sometimes lack the hook at the cloacal region. Occasionally, an anterior localized hook-like structure (with autofluorescence) or hook-like invagination was seen, probably resulting from incomplete 3°-to-2° fate transformation of P9.p. It is not clear if there is any 2°-like fate induction in both P9.p and P10.p of *ras da* animals since following cell lineage is difficult after the heat shock. In a different approach, I built a double mutant between two gain-of-function alleles, *let-23(sa62)* and *let-60(n1046)*, to synergistically increase LET-23/RAS activity. In *sa62; n1046* double mutants, I observed cell division in P9.p and some kind of invaginations around those cells in late L4. However, additional GFP marker expression for ectopic HOA and HOB was not observed (n=14).

The invagination from P9.p descendants usually lacks the characteristics of the hook invagination. Adult males of *sa62; n1046* double mutants were usually Muv and had a misshapen hook, which was often located anteriorly (38/87). In general, the *sa62; n1046* double mutants have similar male Muv and P11-to-P12 transformation phenotypes as *lin-15(lf)* mutants do, although it is unclear whether activated LET-23/RAS signaling in *sa62; n1046* double mutants induces any vulval-like fate in P9.p.

Appendix Table 1. Heat shock treatment in a wild-type background

Genotype[#]	h.s time	stage	abnormality (%)				n
			HOB	hook	spicule	ray	
ceh-26::gfp	20 min	mid L2-early L3	0	0	0	0	5
ceh-26::gfp; hs-mab-5[a]*	20 min	mid-late L2	0	40	80	60	5
		L2 molt-early L3	20	50	90	70	10
	30min	early-late L2	0	60	80	60	5
		L2 molt-late L3	67	87	93	93	15
osm-6::gfp; hs-mab-5[b]*	15min	mid-late L2	n.d.	20	80	20	5
		L2 molt-early L3		72	56	83	18
	20min	mid-late L2	n.d.	33	78	78	9
		L2 molt-early L3		82	65	94	17
	30min	mid-late L2	n.d.	100	100	90	10
		L2 molt-early L3		90	100	100	20

[#] both strains contain *hs-mab-5* transgene *muIs9* (rescues a *unc-31* mutation in the background). The *ceh-26::gfp* transgene is *chIs1200*, while the *osm-6::gfp* transgene is *mnIs17*.

* all have crumpled spicules, at least 10/21 have abnormal rays.

[a] In this strain, overall HOB abnormality observed is 48%, including 16% two-cell GFP expression, 24% absent GFP expression, 4% faint GFP expression, and 4% anterior HOB. Overall hook structure abnormality is 66%, including 20% no hook, 17% misshapen hook, and 29% anterior hook.

[b] In this strain, overall hook structure abnormality observed is 75%, including 30% no hook, 32% misshapen hook, and 13% anterior hook.

Appendix Table 2. Heat shock treatment in a *lin-12(gf)* background

Genotype[#] (heat shock at 33°C, 20 min)	hook structure					n
	0 hook	1 hook			2-3 hook	
		misshapen	anterior	w.t.		
lin-12(gf) /lin-12(null)				1	5	6
*lin-12(gf) /lin-12(null); hs-mab-5**	2	2	3	11	3	21

[#] both strains contain *osm-6::gfp* transgene *mnIs17*. *hs-mab-5* transgene is *muIs9*(rescues a *unc-31* mutation in the background).

* all have crumpled spicules, at least 10/21 have abnormal rays.

Appendix Table 3. Results of cell ablation within HCG

cell(s) ablated	component eliminated	n	phenotype
P9.p & P11.p (late L2)	3° + 1°	2	no hook, no HOB GFP
P9.p & P11.p (early L3)	3° + 1°	5	anterior hook with HOB GFP
P11.p (mid L2)	1°	2	no hook, no HOB GFP or faint HOB GFP
P11.p (early L3)	1°	2	anterior hook, HOB GFP normal
P11.pa & P11.pp	1°	1	anterior hook, HOB GFP normal
P11.pa	part of 1°	3	wild-type
P11.paa	part of 1°	2	wild-type
P11.pp	part of 1°	1	abnormal hook
P11.ppp	part of 1°	4	reduced hook size
P10.pa P10.pap	hook	3 1	HOB GFP ok, no hook
P10.pp & P11.pa P10.pp	HOso + HOB +HOA+HOsh	2 2	no HOB GFP, no hook no HOB GFP, 1 no hook, 1 anterior hook
P10.ppa	HOso + HOB	3	no HOB GFP, 1/3 no hook, 2/3 anterior hook residue
P10.ppaa	HOso	6	HOB GFP ok, 5/6 no hook, 1/6 less autofluorescence
P10.ppap	HOB	2	anterior hook, no HOB GFP
P10.ppp	HOA+HOsh	4	2/4 wild-type, 2/4 anterior hook but HOB GFP ok,
P10.pppa	HOA	5	2/5 anterior hook, 2/5 no HOB GFP, 1/5 faint HOB GFP

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CHAPTER 3

Distinct roles of transcription factors EGL-46 and DAF-19 in specifying the functionality of a polycystin-expressing sensory neuron necessary for *C. elegans* male vulva location behavior

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Distinct roles of transcription factors EGL-46 and DAF-19 in specifying the functionality of a polycystin-expressing sensory neuron necessary for *C. elegans* male vulva location behavior

Hui Yu[1], René F. Prétôt[2], Thomas R. Bürglin[3] and Paul W. Sternberg[1,*]

[1]HHMI and Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA
[2]Division of Cell Biology, Biozentrum, University of Basel, Klingelbergstr. 50/70, CH-4056 Basel, Switzerland
[3]Department of Biosciences at Novum, Karolinska Institutet, Alfred Nobels alle 7, Södertörns Högskola, SE-141 89 Huddinge, Sweden
*Author for correspondence (e-mail: pws@caltech.edu)

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Summary

***Caenorhabditis elegans* polycystins LOV-1 and PKD-2 are expressed in the male-specific HOB neuron, and are necessary for sensation of the hermaphrodite vulva during mating. We demonstrate that male vulva location behavior and expression of *lov-1* and *pkd-2* in the ciliated sensory neuron HOB require the activities of transcription factor EGL-46 and to some extent also EGL-44. This EGL-46-regulated program is specific to HOB and is distinct from a general ciliogenic pathway functioning in all ciliated neurons. The ciliogenic pathway regulator DAF-19 affects downstream components of the HOB-specific program indirectly and is independent of EGL-46 activity. The sensory function of HOB requires the combined action of these two distinct regulatory pathways.**

Key words: Transcriptional regulation, Cell specification, Zinc finger proteins, TEF, RFX factors, Polycystins

Introduction

Because of its simple nervous system with invariant cell lineage and position, *C. elegans* provides an excellent model to study how diverse neuronal subtypes are specified (Sulston and Horvitz, 1977; Sulston, 1983). The anatomy and interconnectivity of all 118 hermaphrodite neuron types are known (White, 1986), as are the molecular details of many neuronal subtypes (Chalfie, 1995). The *C. elegans* male has 79 additional neurons, falling into 37 classes (Sulston et al., 1980). Most of those male-specific neurons are located in the tail region and contribute to specific motor output during mating behavior (Sulston and White, 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995; Garcia et al., 2001; Garcia and Sternberg, 2003).

During mating, the *C. elegans* male scans for the vulva by touching the hermaphrodite with the ventral side of his tail and backing along her body. If the vulva is not found, he turns at the hermaphrodite head or tail and scans the other side (Liu and Sternberg, 1995). The male hook sensillum is a copulatory structure that is located just anterior to the cloaca and mediates vulval location behavior (Liu and Sternberg, 1995). Intact wild-type males usually stop at their first or second vulval encounter. When the hook sensillum is ablated, operated males circle the hermaphrodite multiple times and fail to stop at the vulva (Liu and Sternberg, 1995). This defect is referred to as the Lov (location of the vulva defective) phenotype (Barr and Sternberg, 1999). The hook sensillum consists of five cells, including a structural cell and two ciliated sensory neurons HOA and HOB (Sulston et al., 1980). The two hook neurons have large nuclei and send dendrites into the hook structure; however, their anatomy can be distinguished by cell morphology and synaptic contacts (Sulston et al., 1980). Ablation of either HOA or HOB results in a Lov phenotype, indicating that HOA and HOB have non-redundant functions (Liu and Sternberg, 1995).

The *C. elegans* homologues of human autosomal dominant polycystic kidney disease genes *PKD1* (*lov-1*) and *PKD2* (*pkd-2*) are expressed in the HOB hook neuron (Barr and Sternberg, 1999; Barr et al., 2001; Kaletta et al., 2003). Human PKD genes, which encode divergent members of the TRP family of cation channels, possibly act in signal transduction important for renal epithelial differentiation, as mutations in PKD1 and PKD2 are associated with pathogenic renal cyst formation (reviewed by Wu, 2001). In *C. elegans*, *lov-1* and *pkd-2* mutations disrupt vulva location behavior, consistent with a defect in HOB sensory function (Barr and Sternberg, 1999; Barr et al., 2001). Although LOV-1 and PKD-2 are localized in sensory cilia and cell bodies, the ultrastructure of cilia and dendrites appears normal in *lov-1* and *pkd-2* mutants (Barr et al., 2001).

Another class of genes required for vulva location affects the formation of ciliated endings in sensory neurons. This class includes *che-3*, *daf-10*, *osm-5* and *osm-6* (Barr and Sternberg, 1999). *che-3*, *osm-5* and *osm-6* are required for most or all sensory cilia (Lewis and Hodgkin, 1977; Perkins et al., 1986), while *daf-10* only functions in a subset of ciliated sensory neurons (Albert et al., 1981). The hermaphrodite expression of *osm-5*, a homolog of the mouse autosomal recessive polycystic

kidney disease (ARPKD) gene (Haycraft et al., 2001; Qin et al., 2001), and *osm-6* has been shown to be regulated by a RFX transcription factor DAF-19, which plays a critical role in general sensory cilium differentiation (Swoboda et al., 2000; Haycraft et al., 2001).

We report the isolation of an allele of *egl-46*, a putative zinc-finger transcription factor, in a screen for loci required for fate specification of *C. elegans* hook neuron HOB. *egl-46* was previously characterized as a gene when mutated affecting the development of two mechanosensory neurons (FLP cells) (Wu et al., 2001), as well as having defects in the hermaphrodite HSN egg-laying motoneurons (Desai et al., 1988; Desai and Horvitz, 1989). We demonstrate that EGL-46 and the transcription enhancer factor (TEF) homolog EGL-44 are expressed in the HOB hook neuron and are required for expression of genes encoding polycystins LOV-1 and PKD-2, homeodomain protein CEH-26, and neuropeptide-like protein NLP-8. *egl-44* and *egl-46* mutants are defective in vulva location behavior during mating, suggesting compromised normal HOB function. This HOB-specific pathway is distinct from the DAF-19-regulated general cilia formation pathway in sensory neurons. We found that *daf-19* acts independently of *egl-44* and *egl-46* to affect expression of downstream genes in the HOB-specific program, indicating that general and cell-specific regulatory factors work in concert to establish cell-specific features crucial for HOB neuronal function in sensory behavior.

Materials and methods

Strains

Nematodes were cultured at 20°C as described (Brenner, 1974). All strains used contain *him-5(e1490)* V to obtain males, except for *egl-46(n1127)*, in which case we used *him-8(e1489)* IV (Hodgkin et al., 1979). The following alleles were used in this study: *daf-19(m86)* II (Perkins et al., 1986); *egl-44(n1080)* II, *egl-46(n1127)* V (Desai and Horvitz, 1989); *pha-1(e2123ts)* III (Schnabel and Schnabel, 1990); *unc-119(ed4)* III (Maduro and Pilgrim, 1995); *unc-31(e169)* IV, *unc-46(e177)* V, *dpy-11(e224)* V (Brenner, 1974); *unc-68(e540)* V (Lewis et al., 1980); *unc-42(e270)* V (Riddle and Brenner, 1978); *osm-5(p813)* X (Dusenbery, 1980); and *lin-15(n765ts)* X (Ferguson and Horvitz, 1985). Integrated GFP fusions or extrachromosomal GFP arrays were: *nIs133 (pkd-2::gfp)* I, *nIs128 (pkd-2::gfp)* II (H. Schwartz and H. R. Horvitz, personal communication); *mnIs17 (osm-6::gfp)* V (Collet et al., 1998); *syEx301 (lov-1::gfp)* (Barr and Sternberg, 1999); *myEx256 (osm-5::gfp)* (Qin et al., 2001); *rtEx227 (nlp-8::gfp)* (Nathoo et al., 2001); and *saEx490 (daf-19::gfp)* (Swoboda et al., 2000).

ceh-26 gfp construct

A 6.4 kb fragment of *ceh-26* containing 5277 bp 5′ flanking sequence plus coding sequence to the fourth exon was amplified by long-range PCR using primers P26-22 (GTCCTTTGGCCAATCC-CGGGGATCCAGAGCTACTGTTACTTTCAGGGC) and P26-23 (GCCTGCAGAACATTGGCATGTGGCGTCACGGG). *Bam*HI-digested pPD95.77 was joined to the *ceh-26* fragment by primer extension and linear amplification (Cassata et al., 1998). The product was cut with *Pst*I and circularized to give plasmid pRFP7. pRFP7 (100 ng/μl) was co-injected with *dpy-20* (20 ng/μl) into *dpy-20(e2017)* hermaphrodites as described (Mello et al., 1991). Integration of a transgenic line yielded strain TB1200 with *ceh-26::gfp* integrated transgene *chIs1200* linked to chromosome III. *chIs1200* was crossed into *him-5(e1490)* to yield strain TB1225.

Mapping, cloning, and complementation test

The *sy628* allele was generated by mutagenizing the strain TB1225 carrying the HOB marker *ceh-26::gfp* with EMS using standard protocols (Rosenbluth et al., 1983). In particular, we picked males descended from each single hermaphrodite daughter of mutagenized parents and examined them under a conventional epi-fluorescence microscope for GFP expression. Three-factor mapping of *sy628* on linkage group V used alleles of *unc-46*, *dpy-11*, *unc-68* and *unc-42*: *unc-46* (16/16 recombinants) *dpy-11* (0/16 recombinants) *sy628*; *dpy-11* (0/44) *sy628* (44/44) *unc-42*; *dpy-11* (4/10) *sy628* (6/10) *unc-68*. During the mapping experiments, the presence of *sy628* mutation was determined by loss of *ceh-26::gfp* expression in HOB.

The ~0.6 map unit interval between *dpy-11* and *unc-68* was covered by 17 cosmids, including 97 identified genes or predicted coding sequences (www.wormbase.org, version WS74). The *sy628* hermaphrodites had a mild egg-laying defective (Egl) phenotype. A previously identified gene associated with an Egl phenotype, *egl-46*, is located in the middle of that interval. Cosmid K11G9, which contains the entire *egl-46* locus, was injected into the strain PS3568 *ceh-26::gfp*; *egl-46(sy628) him-5(e1490)* at 40 ng/μl using *pmyo-2::gfp* plasmid pPD118.33 (5.5 ng/μl) as co-transformation marker (Mello et al., 1991). Three stable lines were obtained from individual F1 progeny that expressed *myo-2::gfp* in pharynx. Injection of cosmid K11G9 restored the *ceh-26::gfp* expression in HOB in 76/81 males from three independent transgenic lines. Injection of another cosmid in the same interval, F44C4, which contains a different predicted zinc-finger transcription factor, showed no rescue of HOB expression of *ceh-26::gfp* in fourteen stable transgenic lines (n=172). Those transgenic lines had a non-sex-specific ectopic expression of *ceh-26::gfp* in a neuron anterior to HOB, most likely PVT. It is not clear that this ectopic expression is due to injected F44C4 cosmid or interaction between *pmyo-2::gfp* plasmid and F44C4 cosmid. Cosmids were obtained from the Sanger Institute (Cambridge, UK).

To test for complementation, PS3568 *ceh-26::gfp*; *egl-46(sy628) him-5(e1490)* males were crossed to MT2316 *egl-46(n1127)* hermaphrodites. F1 hermaphrodites with CEH-26::GFP expression were cross progeny, and were examined for an Egl phenotype. F1 males were analyzed by HOB expression of *ceh-26::gfp*. All 79 *sy628/n1127* heterozygous males examined lacked *ceh-26::gfp* expression in HOB, and heterozygous hermaphrodites were Egl. Thus, *sy628* and *n1127* fail to complement.

PCR and sequencing

A 2318 bp genomic DNA fragment containing the entire *egl-46* coding region was PCR amplified from *sy628* mutant DNA using the pair of primers 5′-CTCCCCTTCTTGTAAGGTGTCTT-3′ and 5′-AATTCACTCAGCAATTTGGAAAA-3′. The PCR products from six independent PCR reactions were separately purified using QIAquick PCR purification kit and were pooled together for direct sequencing. Two nested primers, 5′-TTTCGTTCACATCTACCGTAACC-3′ at the 5′ end of the gene and 5′-CGGGGGAAATTGTAAAGAGTTAG-3′ at the 3′ end, and two internal primers, the reverse primer 5′-CCTCTTATGTGCCTTCGTTTTG-3′ at 109-131 bp of the intron 2 and the forward primer 5′-GCTAATGACACCGAGAAAACGAAC-3′ at 274-297 bp of the same intron, were used for sequencing. This sequencing therefore did not cover the 189 bp gap in the intron 2 between reverse and forward primers. The PCR primers and two outside sequencing primers were picked by an oligo design program in the *C. elegans* genome project at the Sanger Institute (www.sanger.ac.uk/Projects/C_elegans/). The two internal sequencing primers were obtained using Maevector software (Oxford Molecular Group). The G-to-A lesion site at nucleotide 165 of the first exon was observed in both strands.

Transgenics

The N-terminal *cfp::egl-46* translational fusion plasmid TU#627 and *yfp::egl-44* fusion plasmid TU#628 were kindly provided by Ji Wu and Martin Chalfie. Plasmid DNAs of TU#627 and TU#628 were

Table 1. HOB gene expression in wild type, *egl-46* and *egl-44* males

Genotype	Marker gene	GFP expression in the HOB neuron			*n*
		Normal	Decreased	Absent	
Wild type	*ceh-26::gfp**	100%			>1000
egl-46(sy628)				100%	>1000
egl-46(n1127)			2%	98%	118
egl-44(n1080)			51%	49%	99
Wild type	*lov-1::gfp*†	93%		7%	113
egl-46(sy628)		1%		99%	92
egl-44(n1080)		21%		79%	75
Wild type	*pkd-2::gfp(nIs128)**	100%			>200
egl-46(sy628)		4%	1%	95%	81
Wild type	*pkd-2::gfp(nIs133)**	100%			>200
egl-46(sy628)		8%	5%	87%	123
egl-46(n1127)		2%	2%	96%	131
egl-44(n1080)		87%	11%	2%	89
Wild type	*nlp-8::gfp*†	96%		4%	98
egl-46(sy628)			49%	51%	97
egl-44(n1080)		77%	16%	7%	87
egl-44(n1080); egl-46(sy628)		17%	59%	24%	93
Wild type	*osm-6::gfp**	100%			>200
egl-46(sy628)		100%			79
egl-44(n1080)		100%			104
Wild type	*osm-5::gfp*†	96%		4%	80
egl-46(sy628)		100%			69
egl-46(n1127)		94%		6%	83
egl-44(n1080)		98%		2%	102

*Integrated transgenes
†Extrachromosomal arrays; a few animals lack expression in HOB because of mosaicism.

injected separately into the strain *unc-119(ed4); him-5(e1490)* at 49 ng/µl. We used 50 ng/µl of pDP#MM016B, a plasmid containing a wild-type copy of the *unc-119* gene, as the co-injection marker. Transgenic animals were recognized by rescue of the Unc phenotype of *unc-119* (Maduro and Pilgrim, 1995). Three independent lines were obtained for each construct and the male expression pattern in those lines was characterized. Transgenic animals generated with the same CFP and YFP plasmids but with *myo-2::gfp* as a transformation marker had similar expression patterns in the male tail.

Mating assay (Vulva location behavior)

The mating behavior of mutant or control males was observed with sluggish *unc-31* adult hermaphrodites. All males were isolated from hermaphrodites at the L4 stage and were kept on fresh plates in groups of ~30 animals before observation. For the mating assay, a virgin adult male (12-36 hr post L4 lethargus) was placed on a 0.5 cm bacterial lawn with five 24-hour-old *unc-31* hermaphrodite adults (Barr and Sternberg, 1999; Garcia et al., 2001). Each individual male was watched under a Zeiss Stemi SV11 or Wild M420 'Macroscope' for ten vulva encounters or until he stopped at the vulva (pausing for more than 1 second or inserting his spicules), whichever came first. The vulva location efficiency of individuals for a population was calculated as described by Barr and Sternberg (Barr and Sternberg, 1999). To facilitate calculation, the vulva location efficiency of males with more than 10 vulva encounters (pass all ten vulva encounters) was considered to be 0 (actual value≤1/11). The Wilcoxon (Mann-Whitney) test was used to determine statistical significance.

Microscopy

GFP expression was analyzed by conventional fluorescence microscopy (Zeiss Axioskop) using a Chroma Technology High Q GFP long-pass filter set (450 nm excitation, 505 nm emission). CFP and YFP were visualized using a Chroma Technology CFP filter set '31044v2' (exciter D436/20, emitter D480/40, beamsplitter 455dclp) and an YFP set '41029' (exciter HQ 500/20, emitter HQ520lp, beamsplitter Q515lp).

Results

Mutations of *egl-46* affect gene expression in the hook neuron and disrupt vulva location behavior in male mating

The male tail is remodeled during the L4 stage, undergoing a series of changes in cell shape and position (Sulston et al., 1980). By the late L4 stage, most of the cells that function in adults reach their final locations, and initiate morphological changes to form the adult tail structures. At this stage, a homeodomain-containing putative transcription factor *ceh-26* (Bürglin, 1994) begins to be expressed and perdures through the adulthood in the HOB hook neuron (Fig. 1A1,A2). Therefore, the presence of CEH-26::GFP indicates a differentiated neuronal fate of HOB. Non-sex-specific expression of *ceh-26::gfp* is mostly in nuclei of the head (R. F. P. and T. R. B., unpublished). To identify genes involved in HOB fate specification, we performed a screen for mutants with altered expression of *ceh-26::gfp* in the HOB cell. This pattern allows for a rapid visual inspection of GFP fluorescence under a compound microscope in the male tail.

One of the mutants recovered from this screen, *sy628*, failed to express *ceh-26::gfp* in the HOB neuron of homozygous males with complete penetrance (Fig. 1A3; Table 1). No effect on non-sex-specific *ceh-26::gfp* expression (e.g., the head nuclei) was observed in *sy628* animals, suggesting that the *sy628* mutation does not cause a general defect in expression of GFP transgenes or of *ceh-26* (data not shown). Anatomical examination of *sy628* males at the third and the fourth larval stages showed that P10.ppap, the presumptive HOB neuron in wild-type animals, was present and occupied its normal position in *sy628* mutants. In addition, the hook structure and overall tail morphology appeared normal under Nomarski

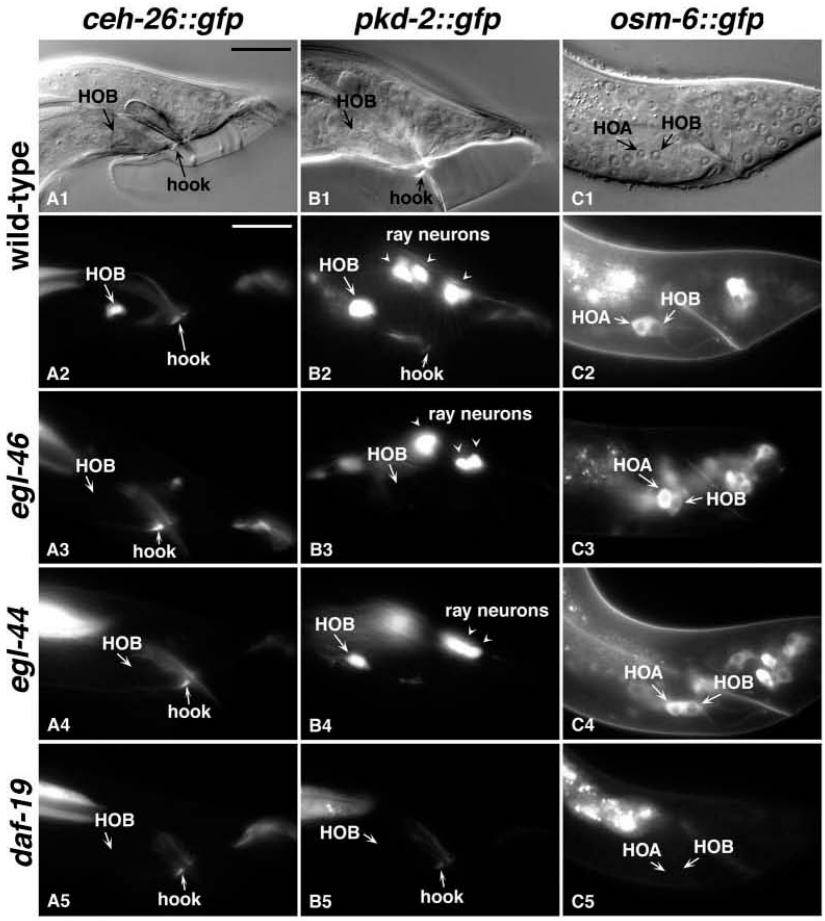

Fig. 1. HOB gene expression in wild-type and mutant males. Left lateral views (anterior leftwards, ventral downwards). Scale bar: 20 μm. (A1,A2) Expression of *ceh-26::gfp* in the HOB neuron of a wild-type adult male. Absence of fluorescence in an *egl-46(sy628)* mutant (A3), an *egl-44(n1080)* mutant (A4) and a *daf-19(m86)* mutant (A5). (B1,B2) HOB and ray expression of *pkd-2::gfp* was observed in a wild-type adult male. (B3) An *egl-46(sy628)* male with ray but not HOB expression. (B4) An *egl-44(n1080)* male with expression in both HOB and ray cells. (B5) No visible expression in both HOB and rays of a *daf-19* mutant. (C1,C2) Normal *osm-6::gfp* expression in HOA and HOB at the L4 stage. Expression was not affected in *egl-46(sy628)* (C3) and *egl-44(n1080)* mutants (C4). (C5) No expression was observed in HOA and HOB cells of a *daf-19(m86)* mutant male. Cell positions of HOB in A3,A4,A5,B3,B5, and HOA and HOB in C5 were located by overlaying with the Nomarski pictures of the same animal. Hook structure autofluorescence is indicated by small arrows. The original *osm-6::gfp* strain has a *ncl-1(–)* background. *ncl-1(–)* was still present in the *him-5* strain of *osm-6::gfp* integrant (C1,C2) and an *egl-46(sy628)* mutant background (C3), but was crossed out in *egl-44(n1080)* (C4) and *daf-19(m86)* mutants (C5). *egl-46(sy628)* mutant also had a *dpy-11* mutation in the background (C3). No effect on *osm-6::gfp* expression was detected for *ncl-1(–)* and *dpy-11* mutations.

optics. Initial observations indicated that *sy628* males had a decreased mating efficiency. Analysis of their mating behavior determined that *sy628* males were deficient in vulval location (the Lov phenotype), but had no obvious defect in other steps of mating, such as response, turning, spicule insertion, or sperm transfer. About 97% of wild-type control males stopped at the vulva during the first two vulva encounters (88% vulva location efficiency), as opposed to only 39% of *sy628* males (Fig. 2A). On average, *sy628* males required more than five encounters to find the vulva, with an overall vulva location efficiency of 36%.

We mapped *sy628* to linkage group V between *dpy-11* and *unc-68*, and identified it as an allele of *egl-46* (see Materials and methods). *egl-46* encodes a putative C2H2-type zinc-finger transcription factor homologous to human and mouse IA1 protein, mouse MLT1 protein and *Drosophila* Nerfin 1 protein (Wu et al., 2001). The lesion in *sy628* mutants was a G-to-A transition at position 165 of the first exon (161-TCTGGAACCCAACGC-175), which changes a tryptophan codon UGG to an UGA opal stop codon. This residue is located at position 55 out of 286 of the inferred EGL-46 protein, before the putative glutamine-rich transcriptional activation domain (residues 61 to 75) and other conserved domains (Wu et al., 2001). This early stop is not necessarily a null allele.

We confirmed the male phenotypes of the *egl-46* mutant using a different allele, *n1127*, which alters the splicing donor of intron 2, located before the region encoding the three zinc fingers of EGL-46 protein (Wu et al., 2001). *n1127* and *sy628* failed to complement (see Materials and methods). Desai and Horvitz (Desai and Horvitz, 1989) found that *n1127* has a decreased male mating efficiency (~50%). We observed that *n1127* males had a Lov phenotype similar to *sy628* mutants (Fig. 2B). The vulva location efficiency of *n1127* males was 39% (n=17), compared with 94% (n=16) for the control males.

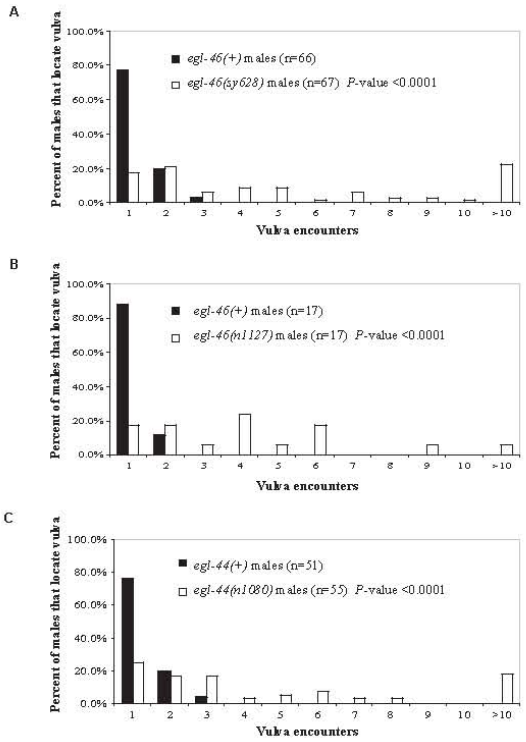

Fig. 2. Vulval location behavior. The *x*-axis represents the number of vulva encounters measured until a tested male stopped at the hermaphrodite vulva. The *y*-axis represents the distribution of males in the tested group that located the vulva at each vulva encounter. (A) *egl-46(+)* versus *egl-46(sy628)*. Both strains have *ceh-26::gfp* III; *him-5(e1490)* V in the background. (B) *egl-46(+)* versus *egl-46(n1127)*. Strains in B contain *him-8(e1489)* IV. (C) *egl-44(+) vs. egl-44(n1080)*. Animals in C are all with *him-5(e1490)* V. In each assay, similar number of wild-type control males and mutant males were examined at same time using the same microscope.

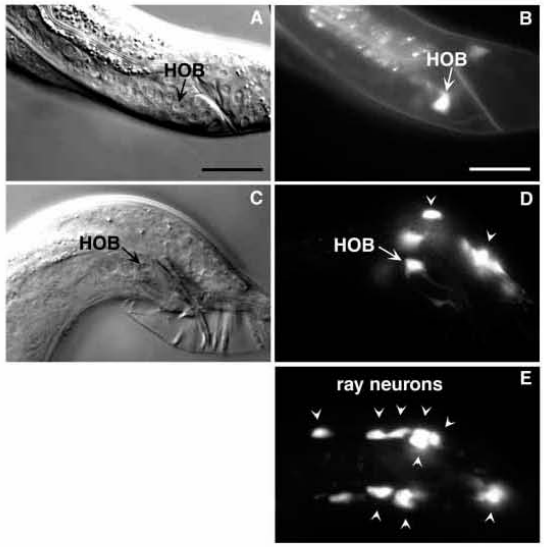

Fig. 3. *egl-46::cfp* expression in HOB and ray neurons of the male tail. Nomarski (A) and fluorescence (B) images of an L4 male with expression in HOB (arrows). Nomarski (C) and fluorescence (D) images of an adult male with CFP expression in HOB (arrows) and ray neurons (arrowheads). (E) Ventral view (left side upwards) of an adult male tail with CFP expression in some ray neurons of both sides (arrowheads). Not all the ray neurons are in the same focal plane. Scale bars: 20 μm. Left lateral views.

There was a marked decrease of *ceh-26::gfp* expression in *n1127* HOB neurons (Table 1). Only two out of 118 *n1127* homozygous males examined retained a faint GFP expression in HOB. No altered expression of *ceh-26::gfp* was detected in cells other than HOB in *n1127* mutants.

egl-46 regulates cell-specific expression of *lov-1* and *pkd-2* to specify the behavioral function of the HOB neuron

The hermaphrodite expression pattern of *egl-46* has been described by Wu et al. (Wu et al., 2001). Using an *egl-46::cfp* construct, we analyzed its expression in males and found a similar pattern for non-sex-specific expression (such as the FLP cells, ventral cord neurons and PVD). Both HOA and HOB are born from a single precursor cell (P10.p) at the late L3 stage, and they differentiate into their neuronal fates during the L4 stage. *egl-46::cfp* was expressed in the HOB neuron beginning at the L4 stage and continuing throughout adulthood (Fig. 3A-D), consistent with the timing of HOB differentiation, and a potential role in the maintenance of HOB function. No detectable expression was seen in the HOA hook neuron. The *egl-46* mating defect is reminiscent of ablation of a hook neuron (Liu and Sternberg, 1995). Based on expression of *egl-46* gene in a single hook neuron, we infer that the Lov phenotype of *egl-46* mutant males is probably due to impaired HOB function.

The dependence of *ceh-26::gfp* expression on EGL-46 activity suggested that the defective HOB sensory behavior caused by an *egl-46* mutation could result from loss of HOB-specific gene expression. The *C. elegans* polycystin genes *lov-1* and *pkd-2* are expressed in HOB and are required for vulva location (Barr and Sternberg, 1999) (Table 1; Fig. 1B1,B2). To test whether *egl-46* regulates these two genes, we used GFP transgenes to visualize their expression in an *egl-46(sy628)* mutant background. *sy628* mutants lacked expression of *lov-1::gfp* in the HOB neuron (Table 1): only one out of 92 animals examined had detectable expression. The expression of *pkd-2::gfp* in HOB was also greatly reduced by the *sy628* and *n1127* mutations of *egl-46* (Table 1; Fig. 1B3). A neuropeptide-like protein-encoding gene, *nlp-8*, is also expressed in HOB as well as in the non-sex-specific neuron PVT in the tail (Nathoo et al., 2001). The PVT expression of *nlp-8::gfp* was not affected by *egl-46* mutations; however the HOB expression of *nlp-8::gfp* was absent in about half of *egl-46(sy628)* males and was decreased in the remainder (Table 1). Therefore, EGL-46 activity is necessary for the HOB expression of all three genes,

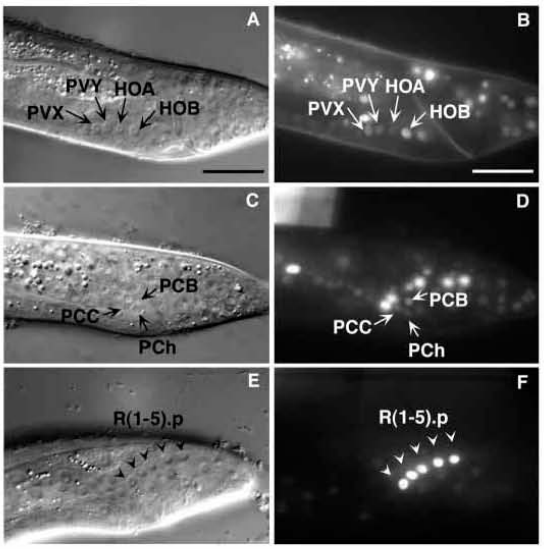

Fig. 4. *egl-44::yfp* expression in the male tail. Left lateral view. Scale bars: 20 μm. (A,B) Different levels of YFP expression in PVX, PVY, HOA and HOB (arrows). In this particular animal, HOA has extremely faint YFP fluorescence (in most cases, YFP expression is undetectable in the HOA hook neuron; data not shown). (C,D) An L4 male with faint YFP expression in the PCB, PCC and PCh cells of the left postcloacal sensilla, in addition to cells from ray lineage in the background. (E,F) Bright expression in hypodermal R1.p, R2.p, R3.p, R4.p, and R5.p at the left side (arrowheads).

and the lack of *lov-1* and *pkd-2* expression could account for the mating defect of *egl-46* mutants.

We also observed male-specific *egl-46::cfp* expression in ciliated ray neurons. The *C. elegans* male has nine pairs of rays (ray 1-9 for both the left and right sides), each associated with an A-type neuron and a B-type neuron (RnA and RnB, *n*=1-9) (Sulston et al., 1980). *egl-46::cfp* was observed in one of the two ray neurons for each ray (Fig. 3D,E); this neuron is probably a B-type neuron because of its co-localization with *pkd-2::gfp* (data not shown), which is known to be expressed in these neurons (Barr and Sternberg, 1999). However, *egl-46* regulation was not necessary for *lov-1* and *pkd-2* expression in ray neurons (Fig. 1B3). *lov-1* and *pkd-2* mutants show deficiencies in both response and vulva location during mating, correlating with their expression in the B-type ray neurons (except ray 6) and the HOB hook neuron (Barr and Sternberg, 1999; Barr et al., 2001). By contrast, despite *egl-46* expression in ray neurons, no obvious defect in either ray neuron expression of PKD genes or response behavior of the mating was detected in *egl-46* mutant males. *egl-46* might play a major role in HOB sensory specification, but some other factors function in ray neurons.

egl-44 exhibits a similar Lov defect for male mutants and may regulate gene expression in HOB

Wu et al. (Wu et al., 2001) reported that *egl-46* acts with *egl-44* to specify subtypes of mechanosensory neurons, and for HSN development in hermaphrodites (Desai and Horvitz, 1989). *egl-44* encodes a transcription enhancer factor of the TEA domain class (Bürglin, 1991) and is orthologous to the mammalian TEF factors (Wu et al., 2001). We therefore examined the behavior of *egl-44(n1080)* males, and found that this *egl-44* mutation reduced vulva location behavior (Fig. 2C). Similar to *egl-46* mutants, *egl-44* mutant males passed the vulva frequently and it took an *egl-44(n1080)* male about five encounters on average to locate the vulva. Specifically, *egl-44* mutant males had an overall 43% vulva location efficiency, while control males (wild-type for the *egl-44* locus) had an 88% vulva location efficiency.

We determined the male tail expression pattern of the *egl-44* gene with the *yfp* construct described by Wu et al. (Wu et al., 2001). Expression of *egl-44* overlapped with but was not identical to that of *egl-46*. At the L4 stage, the four neurons PVX, PVY, HOA and HOB are positioned in a signature anterior-to-posterior row at the middle left side (Sulston et al., 1980). *egl-44::yfp* fluorescence was obvious in HOB, PVX and PVY, with HOB usually the brightest, but was barely visible in HOA (Fig. 4A,B). As stated above, *egl-46::cfp* was only present in HOB. A few neurons anterior to PVX (e.g. PVV) had faint *egl-44::yfp* expression, as did several cells from the B and Y lineage, including PCB, PCC and PCh (Fig. 4C,D). These cells did not express *egl-46::cfp*. In addition, almost all the descendants of the ray precursor cells (Rn) expressed *egl-44::yfp*, including the ray neurons (RnA and RnB) and the ray structure cells (Rnst), all of which are derived from the anterior daughter Rn.a, as well as posterior daughter Rn.p hypodermal cells (Fig. 4E,F; data not shown). EGL-46 showed a more limited expression in the ray lineage. In adults, *egl-44::yfp* was still expressed in HOB, RnA, RnB and Rnst cells. Hypodermal Rn.p cells no longer displayed bright YFP expression in adults, possibly because of their fusion with the tail hypodermal syncytium. Owing to dramatic changes in cell shapes and positions during the extensive male tail remodeling at the L4-adult transition, the faint *egl-44::yfp* expression in PCB, PCC and PCh was hard to trace in adults. Overall, *egl-44::yfp* was expressed more extensively in the male tail than was *egl-46*. However, a mutation in *egl-44* did not result in broader defects in male mating behavior than did an *egl-46* mutation.

Based on its behavioral phenotype and its expression in HOB, *egl-44* might regulate HOB fate specification, similar to *egl-46*. We therefore examined HOB-specific gene expression in an *egl-44(n1080)* mutant background, and found that *egl-44* mutants displayed a significant decrease in HOB-specific expression of *ceh-26::gfp*. 50% (49/99) of *egl-44(n1080)* males lacked *ceh-26::gfp* in HOB, while the remaining 50% (50/99) had weak HOB expression (Fig. 1A4; Table 1). Reduction of *lov-1::gfp* expression in HOB by an *egl-44* mutation was striking, but only a small effect on *pkd-2* and *nlp-8* expression was observed (Table 1; Fig. 1B4). The lesion in *egl-44(n1080)* allele is a missense mutation. It is possible that the residual EGL-44 activity in *n1080* mutants leaded to an incomplete reduction of HOB gene expression. *egl-44* has six differently spliced isoforms (www.wormbase.org, version WS74). The *n1040* mutation affects four of them. Currently, we have no information about which isoform might be dominate in the HOB neuron. Expression of the 'c' form *egl-44* cDNA under control of the 3.1kb *egl-46* promoter gave an ambiguous result, with only about 10% restoration of *ceh-26::gfp* expression in

HOB in each of three transgenic lines (data not shown). *egl-44* mutants were not defective in ray B neuron expression of *lov-1* and *pkd-2*.

Even though the *egl-46* mutations caused a more severe defect in HOB gene expression than did an *egl-44* mutation, the Lov phenotypes are similar in male mutants. One possibility is that incomplete decrease of gene expression in the HOB neuron by the *egl-44* mutation could reduce the HOB function enough to display a comparable Lov phenotype; however, we cannot rule out the possibility that EGL-44 and EGL-46 might have some distinct targets in HOB. In addition, the faint EGL-44 expression in the HOA hook neuron, as well as in the PCB and PCC neurons of the postcloacal sensilla, might also contribute to the vulva location activity (Liu and Sternberg, 1995). The Lov phenotype is not synergistic in the *egl-44; egl-46* double mutant, and there was no observable difference in the efficiency of vulva location compared with single mutants (data not shown). By contrast, *C. elegans* males with HOB ablated have a 0% vulva location efficiency (Liu and Sternberg, 1995). Both *egl-44* and *egl-46* mutants had an incomplete loss of *nlp-8::gfp* expression, but no further elimination of *nlp-8::gfp* expression was seen in an *egl-44; egl-46* double mutant background (Table 1). This lack of enhancement for the Lov phenotype and a defect in *nlp-8* expression indicates that *egl-44* and *egl-46* act at least partially in a common pathway for HOB specification. The *egl-44; egl-46* double mutant males seemed less active than each of the single mutants and took longer to initiate mating behavior, which might be due to insufficient function of the ray neurons in the double mutant.

egl-44 and *egl-46* do not regulate each other's expression in the HOB neuron

In the non-sex-specific FLP cells, wild-type *egl-44* is required for normal *egl-46* expression (Wu et al., 2001). To determine whether *egl-44* and *egl-46* regulate each other's expression in the HOB neuron, we introduced an extrachromosomal *egl-46::cfp* array into an *egl-44* mutant, and an *egl-44::yfp* array into an *egl-46* mutant. The timing and relative brightness of *egl-46::cfp* expression in HOB was not affected in an *egl-44(n1080)* mutant background compared with a wild-type background, but CFP expression in FLP neurons was reduced. Similarly, no change in the HOB expression of *egl-44::yfp* was observed in *egl-46(sy628)* males. We infer that there is no interdependence of *egl-44* and *egl-46* expression in HOB.

The *daf-19* general cilium formation pathway is required for cell-specific features of HOB

Genes that are expressed in HOB and mutate to a Lov phenotype can be grouped into two separate pathways (Barr and Sternberg, 1999) (this work). *osm-5* and *osm-6* belong to a general ciliogenic pathway common to all ciliated neurons, including HOA and HOB (Collet et al., 1998; Qin et al., 2001). The other genes discussed above, including *egl-44*, *egl-46*, *lov-1* and *pkd-2*, define a program specific for HOB differentiation. We thus asked if there are any interactions between these two pathways; i.e., whether regulators in the cell-specific pathway, *egl-44* and *egl-46*, affect the HOB expression of the general cilium structure genes (*osm-5* and *osm-6*), and whether ciliogenesis might be a prerequisite for execution of an HOB-specific program.

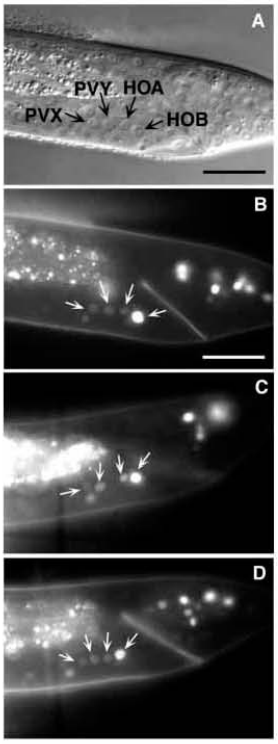

Fig. 5. *daf-19::gfp* expression in the hook neurons. Left lateral view. Scale bars: 20 μm. Nomarski (A) and fluorescence (B) images of a wild-type male tail at the fourth larval lethargus. *daf-19::gfp* expression in HOB is significantly stronger than that in HOA, PVX and PVY. The same expression pattern was present in *egl-46(sy628)* (C) and *egl-44(n1080)* mutant males (D) (arrows).

In wild-type males, OSM-5::GFP and OSM-6::GFP are expressed in the cell bodies and dendrites of HOA and HOB at the late L4 stage; their then expression decreases, which is coincident with the formation of ciliated sensory endings in these two neurons (Collet et al., 1998; Qin et al., 2001). Using an integrated *osm-6::gfp* line (*mnIs17*) and an extrachromosomal array carrying *osm-5::gfp*, we found that the HOB expression of these two GFPs at the L4 stage in *egl-44(n1080)* and *egl-46(sy628)* mutants was comparable with wild-type (Table 1; Fig. 1C1-C4). *osm-5::gfp* expression in HOA and HOB was also not affected by *egl-46(n1127)* (Table 1). In these *egl-44* and *egl-46* mutant males, the HOB dendritic process, visualized by *osm-5::gfp* or *osm-6::gfp*, was extended correctly into the male hook. Neither *egl-44(n1080)* nor *egl-46(sy628)* mutants had dye-filling defects (data not shown). We conclude that mutation of either *egl-44* or *egl-46* impedes neither gross cell morphology nor the ultimate neuronal outgrowth and wiring of HOB.

Qin et al. (Qin et al., 2001) showed that an *osm-5* mutation affects subcellular localization of LOV-1 and PKD-2, but not their expression. We found that *ceh-26::gfp* expression was not affected in *osm-5(p813)* animals. Therefore, it is unlikely that establishment of the HOB-specific program depends on the activities of downstream structure genes (such as OSM-5) in the ciliogenic pathway. The RFX transcription factor DAF-19 is a key upstream regulator of general ciliogenesis (Swoboda et al., 2000; Haycraft et al., 2001). In the male tail, we observed exclusively nuclear-localized GFP expression of *daf-19* in

male-specific ciliated sensory neurons, including the two hook neurons (Fig. 5A,B) and the 36 ray neurons. The fluorescence in HOA was usually fainter than in HOB. We observed no difference in the HOB expression of *daf-19::gfp* in *egl-44* or *egl-46* mutants compared with wild type (Fig. 5C,D). We then analyzed *egl-44::yfp* and *egl-46::cfp* in *daf-19(m86)* mutant males, and found that the timing and relative brightness of expression in HOB was similar to *daf-19(+)* animals. We infer that, during HOB differentiation, *egl-44* and *egl-46* are expressed independently of a general cilium formation pathway governed by *daf-19*.

We next examined the expression of three HOB-specific genes (*ceh-26*, *pkd-2* and *nlp-8*) in *daf-19* mutants. Swoboda et al. (Swoboda et al., 2000) have shown that *daf-19* is required for general cilium formation, but not for cell-specific properties. Surprisingly, *daf-19(m86)* mutants lacked *ceh-26* HOB expression (n=87) (Fig. 1A5). Non-sex-specific expression of *ceh-26::gfp* in some head neurons was also substantially reduced by the *daf-19* mutation. All male-specific expression of *pkd-2* was diminished in the *daf-19* mutant background, including the four ciliated CEM neurons in the head, and the HOB and B-type ray neurons in the tail (n=97) (Fig. 1B5). Only the faint non-sex-specific *pkd-2::gfp* expression in a few neurons posterior to the nerve ring was retained in *daf-19* mutant animals. Similarly, expression of *nlp-8::gfp* in *daf-19(m86)* males was only observed in the non-sex-specific PVT neuron and was totally absent in the HOB neuron (n=91). Therefore, complete execution of the HOB-specific program requires DAF-19 activity.

DAF-19 has been proposed to act on the X-box motifs in the *cis*-regulatory regions of downstream target genes to regulate their transcription (Swoboda et al., 2000). So far, 5′ regions of demonstrated DAF-19 target genes all harbor the X boxes in close proximity to the coding region (the typical spacing is within less than 200 nucleotides upstream). As expected from this hypothesis, expression of X-box-containing *osm-6::gfp* in the hook and ray neurons was not detected in *daf-19* mutants (n=68) (Fig. 1C5). A single X-box sequence is located at about 1.3 kb upstream of the ATG start codon of *egl-46*. This relatively upstream X box in *egl-46* promoter was apparently not a functional target site, as *egl-46::cfp* expression was not altered in *daf-19* mutants. We found no matches to *C. elegans* X-box consensus sequences in the 5′ regions, introns and immediate 3′ regions of *ceh-26*, *lov-1*, *pkd-2* and *nlp-8*. Regulation of *ceh-26*, *pkd-2* and *nlp-8* by *daf-19* is thus likely to be indirect and mediated by some unknown factor(s), which is probably cell-type specific.

Discussion

Specification of the HOB neuron

We have found that *egl-46* is necessary for vulva location behavior, and for gene expression during HOB differentiation. HOB is a ciliated neuron required for *C. elegans* males to sense the vulva during mating (Sulston et al., 1980; Liu and Sternberg, 1995; Barr and Sternberg, 1999; Barr et al., 2001). The regulatory relationships among *egl-46*, another transcription factor, *egl-44*, HOB-specific genes and a ciliogenic pathway support a model involving coordinate contributions of general and cell-specific factors to specify a functional HOB sensory neuron (Fig. 6).

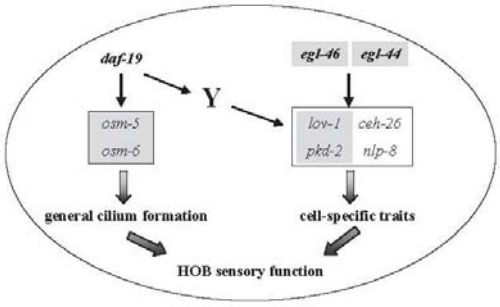

Fig. 6. Distinct pathways involved in HOB gene regulation. Transcriptional regulation by *egl-44* and *egl-46* directs a cell-specific pathway necessary for HOB function in vulva location behavior. In the general ciliogenic pathway, the RFX-transcription factor DAF-19 controls expression of cilium structural genes to provide functional compartment common for all ciliated sensory neurons. DAF-19 has an additional influence on HOB neuronal function by affecting expression of downstream genes in the HOB-specific pathway through some unknown factor(s), indicated by Y. Genes in the shadowed box are the ones in which the Lov phenotype were analyzed in mutants (Barr and Sternberg, 1999) (this work). There are no existing mutants for *ceh-26* and *nlp-8*.

To fulfill its sensory function, HOB must build specific structures and express appropriate molecules to receive and transduce signals. In our model, the general cilium formation pathway governed by *daf-19* programs HOB to have sensory cilia, and *egl-46*, partly with *egl-44*, regulates expression of genes in HOB involved in signal transduction cascades. These two pathways are distinct. Formation of the cilium structures is not necessary for HOB-specific gene expression, and regulators in the cell-specific pathway, *egl-44* and *egl-46*, showed no obvious effect on the HOB expression of the cilium structure genes *osm-5* and *osm-6*. However, these two pathways do interact: not only are they both necessary for HOB function; but the ciliogenic pathway regulator *daf-19* has an effect on downstream components of the HOB-specific program without affecting *egl-44* or *egl-46* expression.

Previous studies suggested that *daf-19* is only required for genes functioning in common aspects of cilium formation (Swoboda et al., 2000). We provide the first evidence that *daf-19* is required for the expression of some cell-type-specific factors. We propose that *daf-19* acts through some unknown factor(s) [which could be an X-box containing gene(s)] to modify HOB-specific gene expression. We observed stronger *daf-19::gfp* expression in HOB than in HOA, but whether it is associated with additional *daf-19* regulation of HOB-specific gene expression is not known. This *daf-19* regulation is not limited to the HOB neuron as *daf-19* also affects *pkd-2* expression in the ray neurons and CEM neurons, indicating some general features are common in this subtype of ciliated sensory neurons. Coupled regulation of general neuronal features and cell-specific identities by multiple transcriptional factors has been found in several different organisms, such as specification of the *C. elegans* AIY interneuron (Altun-Gultekin et al., 2001), *C. elegans* olfactory neurons (Troemel et al., 1997) and vertebrate motoneurons (Novitch et al., 2001;

Zhou and Anderson, 2002), and thus might be a general aspect of the logic of neuronal cell type specification.

Both male hook neurons, HOA and HOB, play a role in vulva location behavior. They both detect the presence of a hermaphrodite vulva, and then produce a distinctive output. This output causes the male to stop at the vulva and to proceed to the next step of mating (Liu and Sternberg, 1995) (M. M. Barr and P.W.S., unpublished). One possible explanation for the functional non-redundancy of HOA and HOB is that they possess different sensory specificity, and hence respond to different cues from the vulva. Another possibility is HOA and HOB might receive the same cues at different times. *egl-44* is broadly expressed in many cells of the male tail, but its expression is almost undetectable in HOA. None of the other genes, including *egl-46* and its downstream targets in the HOB-specific program described here, is expressed in HOA. The unequal expression of those genes in the two hook neurons provides molecular evidence supporting distinct roles for HOA and HOB in mating.

EGL-46 and EGL-44 regulation in HOB sensory function

egl-46 mutations result in an extra cell division in the terminal differentiation of the *C. elegans* Q neuroblast lineage (Desai and Horvitz, 1989). Loss of either *egl-44* or *egl-46* function does not cause a cell division defect or a failure in establishment of primary ciliated neural fate during HOB specification. This was determined by anatomical examination and by expression of the cilium structure genes, *osm-5* and *osm-6*. In the non-sex-specific FLP cells, it has been shown that *egl-44* and *egl-46* act as transcriptional repressors (Wu et al., 2001). They promote the correct subtype of mechanosensory neurons by suppressing expression of genes dedicated to another subtype. Possible positive roles in gene transcription are implicated for *egl-44* and *egl-46* in the HSN neurons, but no target has been identified (Desai and Horvitz, 1989; Wu et al., 2001). Our data suggest a positive effect of *egl-44* and *egl-46* on the expression of downstream HOB-specific genes. However, we have not ruled out that EGL-44 and EGL-46 activate gene expression in HOB by repression of a repressor of HOB-specific genes.

We propose that the sensory abilities of the HOB neuron are established by individual cell-specific components regulated by *egl-44* and *egl-46*. One of these components, *ceh-26*, is the *C. elegans* ortholog of *Drosophila prospero* (*pros*) gene (Bürglin, 1994). *pros* is involved in the initiation of differentiation in specific neurons following asymmetric cell division (Hirata et al., 1995; Broadus et al., 1998; Manning and Doe, 1999). However, expression of *ceh-26* in HOB is not coupled with cell division. Instead, it is expressed at a much later stage, after basic features of cell fate have been established. Similar to HOB, ray B neurons express both *egl-44* and *egl-46*, but unlike HOB, these neurons do not express *ceh-26::gfp*. Therefore, we think that co-expression of *egl-44* and *egl-46* is not sufficient to activate *ceh-26::gfp* in HOB and additional co-factors are also required. The other downstream components, *lov-1*, *pkd-2* and *nlp-8*, encode proteins that are probably involved in HOB sensory input and output. LOV-1 and PKD-2 accumulate in the sensory cilia and have been proposed to act in a complex; a working model is that LOV-1 is a sensory receptor and PKD-2 is a channel protein (Barr et al., 2001; Koulen et al., 2002). Neuropeptide-like protein NLP-8 might act as a neurotransmitter or neuromodulator released by HOB to mediate the response to the stimuli from the hermaphrodite vulva.

Potential mechanosensory and chemosensory interactions between the male and the hermaphrodite during mating is implied by the vulva location behavior itself, as well as by the requirement of functional ciliated sensory endings in the two hook neurons. Whether HOB is a mechanical sensor or a chemical sensor or both, as is the case for the polymodal ASH neuron (Kaplan and Horvitz, 1993), is not known. Because *egl-44* and *egl-46* distinguish between mechanosensory neuron subtypes during FLP fate specification, it is possible that these two genes regulate downstream targets that confer mechanosensory ability to the HOB neuron. If so, as members of TRP protein gene family, *lov-1* and *pkd-2* might be such targets. Known examples of TRP proteins that play a role in mechanotransduction include a *C. elegans* TRP protein OSM-9 and the *Drosophila* TRP-like NOMPC protein (Colbert et al., 1997; Walker et al., 2000). Both of these TRP proteins are expressed in mechanosensory neurons and are involved in mechanosensory response.

Transcriptional regulation of polycystins and polycystic kidney disease

Human PKD1 and PKD2 were identified as two loci responsible for the autosomal dominant polycystic kidney disease (ADPKD), a genetic disorder that causes renal failure at various ages of adulthood (reviewed by Gabow, 1993; Wu, 2001). Relatively little is known about the regulation of these PKD genes and possible alterations during the disease process. In this work, we showed that expression of *C. elegans* PKD gene homologs, *lov-1* and *pkd-2*, is affected by transcription factors *egl-44* and *egl-46*. The mammalian TEF proteins, homologous to *egl-44*, have been implicated in multiple developmental processes (Chen et al., 1994; Jacquemin et al., 1996). Specific expression in kidney was reported for multiple members of TEF proteins (Jacquemin et al., 1996; Kaneko et al., 1997; Jacquemin et al., 1998). *C. elegans* EGL-46 belongs to a novel zinc-finger protein subfamily. Identified close mammalian homologs of *egl-46* includes insulinoma associated (IA) proteins, implicated in islet differentiation of the pancreas, and murine MLT 1 protein, silenced in the liver tumors (Goto et al., 1992; Tateno et al., 2001), but their possible roles in the kidney have not been investigated. Progressive cyst formation in ADPKD is not restricted to kidney: involvement of the liver and the pancreas occurs, indicating that those organs suffer similar pathogenesis during progression of the disease (Gabow, 1993; Chauveau et al., 2000). The demonstrated gene regulation network in HOB might reveal important insights into the regulation of human polycystin gene expression.

The dependence of ciliogenesis for the function of PKD-2 may be even more relevant to renal development in mammals. In *C. elegans*, the ARPKD homolog *osm-5* is a direct target of the RFX factor DAF-19 (Haycraft et al., 2001), making the requirement of DAF-19 activity for *pkd-2* expression particularly interesting with regard to the link between ADPKD and ARPKD. Mammalian polycystins and the cilia of the kidney cells might participate in a common signaling pathway crucial for renal differentiation and function. This

hypothesis implies that RFX factor(s) might play a role in the renal development.

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APPENDIX

Four additional mutants isolated from *ceh-26::gfp* expression screen

In addition to the *egl-46(sy628)* mutant, I identified four other mutants with distinct phenotypes. *sy630* males lose *ceh-26::gfp* expression in HOB with ~50% penetrance and have short blobby rays. This mutant is mapped to linkage group (LG) I between *dpy-5* and *unc-29*, and is probably very close to the *unc-29* locus. Another mutant, *sy645*, shows pleiotropic hook sensillum defects: ~ 50% of animals lack *ceh-26::gfp* expression in HOB; the hook structure can be misshapen or located anteriorly or even absent. Occasionally, a *sy645* male has two rudimentary hooks and/or two *ceh-26::gfp*-expressing HOB cells, indicating additional 2° HCG fate formation. *sy645* males look Mab in general with missing (or fused) or blobby rays, reduced fan, and a few of them also have crumpled spicules. Different individuals have different combinations of these Mab phenotypes. Other phenotypes of *sy645* mutants include small, dumpy, uncoordinated, slow growth, Egl (egg-laying defective), and low brood size. I mapped *sy645* to LG III between *dpy-17* and *unc-119*, closer to *dpy-17*. The third mutant, *sy641*, exhibits decreased *ceh-26::gfp* expression in HOB and might have some defects in mating. This mutant has a slightly protruding vulva in hermaphrodites. The fourth mutant is *sy642*, which lacks sclerotic cuticle on the surface of the hook as indicated by absence of yellowish autofluorescence. Preliminary mapping data have failed to localize *sy641* and *sy642* to the cluster of any chromosome.

egl-44 site of action

To test the autonomy of *egl-44* function for *ceh-26:gfp* expression, we expressed *egl-44* under the control of the *egl-46* promoter since we have shown that *egl-46* expression in HOB is not dependent on *egl-44*. In particular, 3.1kb *egl-46* 5' upstream region was amplified by PCR with a Pst I site at the 5' end and an Spe I site at the 3' end, and was cloned into the promoterless vector pPD49.78 from Andy Fire using Pst I and Xba I sites in the vector. A C-form of an *egl-44* cDNA was excised from the clone *yk1261h07* (kindly provided by Yuji Kohara, National Institute of Genetics) using Nco I and Pst I sites on the pME18sFL3 vector (the Pst I site was fill-ined to create a blunt end), and inserted into the pPD49.78 vector under the *egl-46* 5' region by Nco I and EcoR V sites. I injected the construct into an *egl-44(n1080)*; *ceh-26::gfp* background, using *myo-2::gfp* as a transformation marker. Of the eleven stable lines, I analyzed the three with the highest transmission rate. These lines had ~10% restoration of *ceh-26::gfp* expression in HOB, but only one consistently had this ~10% rescue in subsequent generations. We think that this result is too marginal to be considered as positive. One possible explanation is that the construct used to determine the *egl-46* expression pattern not only contains 3kb of upstream sequence but also includes all the introns and exons (Wu et al., 2001), and it is not clear whether additional regulatory elements exist in those regions. Also, *egl-44* has five differently spliced isoforms; the cDNA used here is a C-form clone (WS 128). The C-form differs from the B-form in that it lacks the last 7 amino acids at the end of the exon 4 (the *n1080* lesion is at the beginning of the exon 4). The A-form has an additional exon further upstream. The D-form and F-form starts at the exon 3 and 4 of B/C-form, respectively. The E-form is a short one, starting at the middle of exon 6 with a frame shift, therefore joining the exons 6 and 7 together. The published *egl-46*

sequence is a combination of the C and E forms (Wu et al., 2001). We currently have no information about which isoform might predominate in the HOB neuron. To test the rescue rigorously will require testing each cDNA and perhaps their combinations. We did not use *egl-44* genomic DNA in this ectopic promoter assay since the actual gene expression pattern could be questionable when all the introns are still present.

ceh-26 RNAi

To examine the possible regulatory role of *ceh-26* on HOB gene expression, I tried to disrupt *ceh-26* function by RNA-mediated interference (RNAi) (Fire et al., 1998). The ORF of the *ceh-26* gene contains ten exons (WS 128). The seventh exon (472bp) is the biggest one. A ~360bp fragment of the seventh exon was PCR amplified and head-to-head ligated to generate inverted dimers. I added the fold-back piece of *ceh-26* exon 7 under the *hsp-16.2* promoter (pPD49.83) to make a heat-shock inducible RNAi construct but was unable to obtain stable clones in multiple attempts, although a special bacteria host was used (Tavernarakis et al., 2000). I then tried dsRNAi by soaking (Tabara et al., 1998), in which the exon 7 fragment was inserted into pBluscript vector by Bam HI and Spe I sites and dsRNAs were obtained by *in vitro* transcription using T3 and T7 promoters in the vector. L1 larvae of strain PT31 (*pkd-2::gfp*) and TB1225 (*ceh-26::gfp*) were soaked in the *ceh-26* exon 7 dsRNA at 1μg/μl for 24 hours but no effect on expression of either *ceh-26::gfp* or *pkd-2::gfp* was found. *ceh-26* functions in HOB at a relatively late developmental stage and in a neuronal environment. Both conditions are not sensitive to RNAi treatment (Tavernarakis et al., 2000).

Other hook neuron markers

We found that a degenerin homologue *T28F4.2* was expressed in HOB, ray 4, and ray 9 in adult males (the *T28F4.2::gfp* transgene was kindly provided by Monica Driscoll, Rutgers University). The *egl-46(sy628)* animals lost *T28F4.2::gfp* expression in HOB completely and in about one-third of males in ray 4 and ray 9 (n=94). In *egl-44(n1080)* mutants, the male-specific *T28F4.2* expression was down-regulated similarly to what was seen in *sy628* males (n=106). EGL-44, mammalian TEF proteins, and *Drosophila* TEF (Scalloped) are highly similar in their conserved TEA/ATTS DNA-binding domains (Wu et al., 2001), indicating that EGL-44 might bind to the same consensus DNA sequence (A/T)(G/A)(G/A)(A/T)ATG(T/C) revealed by studies on human and murine TEFs (Jiang et al., 2000). There are five potential TEF consensus sequences in the *ceh-26* 5' region, three each in the *lov-1* and *nlp-8* 5' region, two in the *pkd-2* 5' region, and six in the *T28F4.2 5'* region. It is not known whether such consensus sequences are the actual *egl-44* binding sites. Interestingly, when *ceh-26::gfp,* which is a translational fusion, was crossed into the strain carrying *T28F4.2::gfp,* we noticed an increased intensity of *T28F4.2::GFP* (data not shown), suggesting that *T28F4.2::gfp* expression could be regulated by *ceh-26*. As a member of *C. elegans* degenerin protein family, *T28F4.2* might encode a signal receptor. It has been shown that the degenerin /epithelial sodium channel (DEG/ENaC) proteins form homo- or hetero-multimers functioning in mechanosensation, acid perception, and epithelial Na+ absorption (Huang and Chalfie, 1994; Price et al., 2000; Snyder et al., 1995; Tavernarakis et al., 1997; Waldmann et al., 1997).

unc-119 is a neuronal structural gene and is expressed in many neurons of the male tail (Maduro and Pilgrim, 1995). The expression of *unc-119::gfp* is seen during late L4 in PVX, PVY, HOA, and HOB, which form a linear array. However, expression in HOA is very low as GFP is usually very faint or even undetectable.

dgn-2 (F56C3.6) encodes a dystroglycan-like protein. It might be expressed in HOA (James Kramer, personal communication). I observed a *dgn-2::gfp*-expressing cell before the cloaca in some adult males. Based on the position, the cell could be the HOA hook neuron. However, the *dng-2::gfp* transgene *cgIs18* (a gift from James Kramer, Northwestern University) is not integrated and this HOA-like expression is quite mosaic, which might impede verification of the cell identity by laser ablation.

Search of neurotransmitters released by HOB and/or HOA

We examined possible neurotransmitters released by HOA and HOB using GFP reporter constructs for the genes *unc-17*, *eat-4*, *tph-1*, and *cat-4*. *unc-17* is a vesicular acetylcholine transporter gene, *eat-4* encodes a sodium-dependent phosphate cotransporter necessary for glutamatergic transmission, *tph-1* encodes the enzyme tryptophan hydroxylase required for serotonin biosynthesis, and *cat-1* is homologous to the mammalian vesicular monoamine transporter genes and is required for dopamine-mediated sensory behaviors (Alfonso et al., 1993; Duerr et al., 1999; Lee et al., 1999; Sze et al., 2000). Expression of each gene corresponds with the neurotransmitter released by the neuron. Based on GFP reporter studies, none of those four genes are expressed in the HOA and HOB neurons (L. R. Garcia, M. M. Barr, H. Yu and P. W. Sternberg,

unpublished data). It is possible that HOB and/or HOA utilize neuropeptides for sensory output (e.g. *nlp-8*). On the other hand, *eat-4* and *cat-1* display some male-specific expression. As mentioned earlier in the Chapter 2, *eat-4* is expressed in PVV, the most anterior progeny of P11.p, making *eat-4::gfp* a 1° marker. The cytoplasmic punctate expression of *cat-1::gfp* was observed in both the anterior-most three cells and the posterior-most three cells of the vas deferens. It is unclear whether *cat-1* has a role in coordination of sperm transfer (or release) during mating. *cat-1::gfp* was also expressed in the posterior edge of the gubernaculum (B.paa), two cells from the spicule sensilla (sheath or socket cells?), as well as one neuron (A-type) of three pairs of rays, ray 5, 7, and 9.

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CHAPTER 4

Summary

Signaling pathways such as EGF-Ras, Wnt, and Notch pathways are conserved between species and control many processes during the development of multicellular organisms. This raises an interesting question about how common signaling cassettes regulate various processes that have distinct outputs, and where the specificity comes from. One part of the answer could reside in the diversity and complexity of signaling itself. Multiple signaling pathways could merge to produce a unique developmental outcome, while the same type of signaling may have alternative components in different processes. Another source of specificity may be the particular effectors that are available in each system to respond to the signal.

With a completely sequenced genome and powerful genetic and molecular tools for manipulation, *Caenorhabditis elegans* makes an excellent model system for functional studies of conserved signaling pathways. The well-characterized hermaphrodite vulval precursor cells (VPCs) provide a nice example of how carefully orchestrated integration of multiple signaling pathways controls specific pattern formation. The *C. elegans* hook sensillum competence group (HCG) is the male homolog of the hermaphrodite VPCs based on the origin and the three-fate setting. However, unlike vulval induction, signals involved in HCG patterning have remained a mystery. The research presented in this thesis demonstrates that Wnt signaling is the major player in HCG patterning. This finding builds the foundation for a more detailed investigation of the molecular mechanism of pattern formation during male hook development. As the other part of my work, I looked into gene regulation in one of the HCG progeny, the HOB hook neuron, and the effects on its neuronal identities and functions in mating behavior. The progress

described in the Chapter 3 takes a step forward in our understanding of the specification of neuronal subtypes and the genetic basis of stereotyped behavior. In the following parts, I will briefly discuss future research directions.

The inductive Wnt signal

To understand the molecular mechanism of HCG patterning, it is critical to identify the signals required to establish this spatial pattern. Mutation in either of the three Wnt ligands, *egl-20, lin-44,* and *mom-2,* causes no defect in hook formation. It remains to be tested whether *cwn-1* or *cwn-2* is the Wnt ligand triggering the HCG fates. Multiple Wnt molecules might signal redundantly to pattern the male HCG. It is also possible that distinct Wnt ligands function at different steps of HCG patterning. Further analysis of the double and triple mutants of different Wnt ligands will provide us with more details about the inductive signal in male hook sensillum development.

LIN-17 time of action

The *lin-17* mutant is one of the three mutants that have been identified so far with a hookless phenotype, and is the only one that exhibits defects in both the 1° and the 2° HCG lineages. The other two hookless mutants are the *mab-5* loss-of-function mutant, in which P(9-11).p abnormally fuse with hyp7 prior to HCG patterning (Kenyon, 1986), and the *lin-12* loss-of-function mutant, in which the 1° fate of P11.p is not affected and its hookless phenotype is due to a failure of 2° fate specification in P10.p (Greenwald et al., 1983; see Appendix of the Chapter 2). One outstanding concern is whether *lin-17* is the major receptor that mediates Wnt signaling during HCG patterning. Current results we

have are not conclusive about which step of HCG pattern formation is affected by deficient LIN-17 function. The LIN-17 pathway is probably dispensable for HCG competence, as P10.p and P11.p remain proliferative in most *lin-17* mutants. However, we cannot rule out that the LIN-17 activity partially contributes to this step. The expression pattern of *lin-17::gfp,* and the disrupted *bar-1::gfp* expression in P11.p of a *lin-17* mutant, imply a role for *lin-17* in specifying the 1° HCG fate. The phenotypes of a *lin-17* single mutant, a *lin-17; lin-12(gf)* double mutant, and a *lin-17; mab-5(gf)/lin-12(gf)* triple mutant are consistent with the requirement for *lin-17* in HCG fate execution. In principle, a *mab-5* gain-of-function mutation should bypass the need for Wnt signaling in the acquisition of competence, and a *lin-12* gain-of-function mutation produces the 2° hook independent of the 1° fate. Therefore, the failure to rescue *lin-17(lf)* hookless phenotype by a combination of *mab-5(gf)* and *lin-12(gf)* strongly indicates a defect in HCG fate execution. However, there is a possibility that overexpression of *mab-5* by the gain-of-function mutation is still LIN-17-dependent, which then means that *lin-17* may be necessary for HCG competence (of which proliferation is just one aspect), and that defects of *lin-17* mutants could be due to insufficient HCG competence and induction. To address this issue, a *hsp::mab-5* transgene *muIs9* (Salser et al., 1993) can be introduced into a *lin-17* mutant or a *lin-17; lin-12(gf)* double mutant to examine if LIN-17-independent *mab-5* expression by heat shock is capable of rescuing the hookless defect in these two mutants. Given that too much *mab-5* activity at a later stage disturbs hook formation (see Appendix of the Chapter 2), the heat shock treatment would have to be performed in the early L2 stage.

The best way to clarify the role of *lin-17* during HCG patterning is to temporally perturb *lin-17* function. One method is to express *lin-17* under a heat shock promoter in the *lin-17* mutant to see in which developmental period heat shock-activated gene expression rescues the *lin-17* defects. Another method is to screen for temperature-sensitive conditional alleles of *lin-17* and then use temperature shift assay to determine the LIN-17 time of action. Alternatively, heat shock-induced *lin-17* RNAi or overexpression of a dominant-negative form of LIN-17 protein that lacks the intracellular domain can also be used to manipulate LIN-17 activity in different developmental stages. In addition, it may still be worth the effort to figure out whether *Y73B6BL.21* can work as a true Wnt signaling antagonist. Therefore the *Y73B6BL.21* cDNA, instead of the genomic coding region, could be driven by the heat shock promoter and then tested for effects of *Y73B6BL.21* overexpression on Wnt signaling in vivo.

Other Wnt pathways

The P10.p and P11.p cell divisions and residual hook formation in *lin-17* mutants suggest that a second signaling pathway acts in HCG patterning. Three other Frizzled-like receptors, MOM-5, MIG-1, and CFZ-2, and a LIN-18 RTK receptor might mediate Wnt signaling in parallel to LIN-17 (Inoue et al., 2004; Korswagen et al., 2002). Most interestingly, *lin-18* has an expression pattern reciprocal to that of *lin-17* in P10.p and P11.p, which makes *lin-18* a good candidate for the second Wnt receptor. The *lin-17* and *lin-18* pathways may work in concert to pattern the male HCG in response to different Wnt ligands, similar to what has been demonstrated in P7.p VPC development (Inoue et al., 2004). My preliminary analysis of a *mig-1* mutant found no defect in hook formation.

It remains to be examined if a *mom-5* mutation or *cfz-2* RNAi causes any abnormality in HCG patterning. The double or triple mutants of *lin-17* with *lin-18* and other *C. elegans* Frizzled-like receptors can be obtained using a combination of genetic construction and RNAi. It will be interesting to see whether the proliferation and differentiation of P(10-11).p lineages are completely abolished in these males.

The *bar-1* expression pattern and the *pry-1* mutant phenotype are consistent with a role for *bar-1* in the 1° HCG fate specification. However, no sign of 1° fate disruption is seen in *bar-1* mutants. A mutation in a different β–catenin *wrm-1* causes similar defects in hook formation, and also shows no effect on the 1° fate, indicating that these two β–catenins may share tasks during HCG pattern formation. It is important to find out if the 1° HCG fate is affected in a *wrm-1; bar-1* double mutant.

Our results suggest that *lin-17* is required in the specification of the 1° HCG fate and the execution of both the 1° and 2° fates. Whether the β-catenin pathway regulates HCG fate execution is unknown. It is also possible that non-canonical Wnt pathways function synergistically with the β-catenin pathway. In *Drosophila*, the Dishevelled protein acts between the Wnt receptor and downstream components, and can be involved in both the canonical Wnt pathway and the planar polarity pathway (Boutros et al., 1998). *C. elegans* has three Dishevelled homologs, MIG-5, DSH-1 and DSH-2 . It has been shown that PRY-1 physically interacts with MIG-5 but not with DSH-1 and DSH-2 (Korswagen et al., 2002). The possible function of these Dishevelled genes during male hook development can be investigated by RNAi experiments. These RNAi phenotypes can

also be compared with the hook sensillum defects in β-catenin mutants to determine if there is a potential *C. elegans* polarity pathway operating in male HCG fate execution. Another conserved non-canonical Wnt pathway is the Wnt/Ca++ pathway, in which PKC is a major component (Kuhl et al., 2000). Among three *C. elegans* PKC homologs, *pkc-3*, when mutated, has a protruding vulva phenotype in hermaphrodites (Kamath et al., 2003), implying that Ca++ signaling influences some aspects of vulval development. The male tail phenotype will be examined in different *pkc* genes to elucidate whether a Ca++ pathway plays a part in HCG patterning.

Interaction of LIN-17 with the EGF pathway

The second pathway in HCG patterning might come from a non-Wnt signal. In the posterior part of the *C. elegans* body, EGF signaling regulates P11/P12 decision and male spicule development (Chamberlin and Sternberg, 1994; Jiang and Sternberg, 1998). The severe reduction-of-function mutants in the EGF signaling pathway showed no obvious defects in HCG fate specification. However, the role of EGF signaling might be underestimated due to the dominant function of Wnt signaling and lack of viable null mutants of major components in EGF signaling. The 2°-like fate induction in P9.p of a *lin-15* mutant and ectopic HCG fate formation in anterior P(1-8).p of a *mab-5(gf); lin-15* double mutant suggest that EGF signaling is able to induce the HCG fate in competent cells, raising a possibility that EGF signaling assists Wnt signaling during HCG fate induction. A strong reduction-of-function allele of *let-23* (EGFR), *sy97*, has an abnormal male tail phenotype (although no hook defect) (Chamberlin and Sternberg, 1994), and thus can be used to construct a double mutant with *lin-17* to test if *let-23(sy97)* enhances

the *lin-17* mutant phenotype in the male HCG, resulting in 3°-like undivided P10.p and P11.p.

Terminal differentiation of the 2° HOB neuron

Generation of a functional hook sensillum that mediates vulva location behavior during mating requires precise execution of the 2° HCG lineage. All major components of the hook sensillum, including a structural cell, two supporting cells, and two sensory neurons, are descended from the 2° P10.p lineage (Sulston et al., 1980). The terminal differentiation of the hook sensillum involves final morphogenesis and production of cell-specific features. Our studies presented in the Chapter 3 depict a transcriptional regulatory cascade in the hook neuron HOB, demonstrating how general and cell-specific regulators coordinate to establish general neuronal traits and cell-specific identities necessary for HOB sensory function in mating behavior. Among these transcriptional regulators, an RFX factor *daf-19* is particularly interesting due to its dual function in regulating both a general ciliogenic pathway and a HOB-specific program. Intriguingly, none of the genes in the HOB-specific program contains DAF-19 binding sites in their genomic regulatory regions, indicating that DAF-19 acts through some unknown factor to indirectly regulate HOB-specific gene expression. Theoretically, this unknown factor should contain an X-box consensus of the DAF-19 binding site within less than 200bp upstream of the coding region, as observed in other DAF-19 target genes (Swoboda et al., 2000). Using bioinformatic tools, candidate DAF-19 targets can be identified in the *C. elegans* genome. Candidate genes with the most interesting homologies, such as transcription factors or molecules participating in signal transduction, could be further

analyzed to determine if any one of them is the missing link between DAF-19 and HOB-specific genes. A human autosomal recessive polycystic kidney disease (ARPKD) homolog *osm-5* is a DAF-19 direct target in the general ciliogenic pathway, and two autosomal dominant polycystic kidney disease (ADPKD) homologs *lov-1* and *pkd-2* are part of the HOB-specific program (Barr et al., 2001; Barr and Sternberg, 1999; Haycraft et al., 2001). Therefore, an understanding of the regulatory cascade governed by DAF-19 may reveal some common mechanism involved in progression of these two types of polycystic kidney diseases.

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