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Wnt and EGF pathways act together to induce *C. elegans* male hook development

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Running title: *C. elegans* male HCG fate specification

Abstract

Comparative studies of vulva development between *Caenorhabditis elegans* and other nematode species have provided some insight into the evolution of patterning networks. However, molecular genetic details are available only in *C. elegans* and *Pristionchus pacificus*. To extend our knowledge on the evolution of patterning networks, we studied the *C. elegans* male hook competence group (HCG), an equivalence group that has similar developmental origins to the vulval precursor cells (VPCs), which generate the vulva in the hermaphrodite. Similar to VPC fate specification, each HCG cell adopts one of three fates (1°, 2°, 3°), and 2° HCG fate specification is mediated by LIN-12/Notch. We show that 2° HCG specification depends on the presence of a cell with the 1° fate. We also provide evidence that Wnt signaling via the Frizzled-like Wnt receptor LIN-17 act to specify the 1° and 2° HCG fate. A requirement for EGF signaling during 1° fate specification is seen only when LIN-17 activity is compromised. In addition, activation of the EGF pathway decreases dependence on LIN-17 and causes ectopic hook development. Our results suggest that WNT plays a more significant role than EGF signaling in specifying HCG fates, whereas in VPC specification EGF signaling is the major inductive signal. Nonetheless, the overall logic is similar in the VPCs and the HCG: EGF and/or WNT induce a 1° lineage, and LIN-12/NOTCH induces a 2° lineage. Wnt signaling is also required for execution of the 1° and 2° HCG lineages. *lin-17* and *bar-1*/β-catenin are preferentially expressed in the presumptive 1° cell P11.p. The dynamic subcellular localization of BAR-1–GFP in P11.p is concordant with the timing of HCG fate determination.

Introduction

The development of multicellular organisms often involves the specification of different fates among a set of similarly multipotent cells called an equivalence group (Campos-Ortega and Knust, 1990; Carmena et al., 1995; Eisen, 1992; Kelley et al., 1993; Kimble, 1981; Lanford et al., 1999; Weisblat and Blair, 1984). Cells of an equivalence group possess similar developmental potentials but adopt different fates as a consequence of cell-cell interactions. Comparative studies of the patterning of equivalence groups help us to understand the evolution of the cellular and genetic networks responsible for the specification of cell fates among members of an equivalence group. One well-studied example of cell patterning is vulval precursor cell (VPC) specification. In *C. elegans*, each postembryonic Pn (n=1, 2, 3, …, 12) precursor cell, located ventrally along the anterior-posterior axis, divides once to produce an anterior (Pn.a) and a posterior daughter (Pn.p) during the first larval (L1) stage (Sulston and Horvitz, 1977). In hermaphrodites, the six central Pn.p cells, P(3-8).p, constitute the VPC equivalence group. The VPCs can adopt one of three vulval fates (1°, 2° or 3°) and exhibit a spatial pattern 3°-3°-2°-1°-2°-3° in response to an inductive signal from the gonadal anchor cell (AC) (Kimble, 1981; Sternberg, 2005; Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977). The vulva is formed from the descendants of the 1° P6.p lineage, which is most proximal to the AC, and the 2° P5.p and P7.p lineages. The more distant P4.p and P8.p cells acquire the 3° fate, while P3.p adopts either the 3° or the F fate (which is to fuse with the hyp7 epidermal syncytium without dividing in the L2 stage, prior to induction). Wnt and EGF signaling are required during the L2 stage, to prevent P(4-8).p from fusing to hyp7 (Eisenmann et al., 1998; Myers and Greenwald, 2007). The 1° fate is induced by EGF signaling and the Wnt pathway appears to play a lessor role in induction (Eisenmann et al., 1998; Sternberg and Horvitz, 1986). Subsequently, the 1° cell signals laterally to promote the 2° fate and prevent it from acquiring the 1° fate (Greenwald et al., 1983).

Studies of other nematodes such as *Oscheius*, *Rhabditella* and *Pristionchus* have provided us with some insights into the evolution of vulva development and demonstrated that the use and importance of different cell-patterning mechanisms in vulval development vary among nematode species (Felix and Sternberg, 1997; Sommer, 2005). However, studies of species other than *C. elegans* describe these patterning mechanisms in terms of the source of induction and the number of induction steps required, and molecular details are known only for *Pristionchus pacificus*, for which it has been shown that EGF signaling does not seem to be required for induction, while Wnt signaling has a more important role in vulval development (Tian et al., 2008). Certain Wnt components are required for induction (Tian et

al., 2008) while others have a repressive role (Zheng et al., 2005). Fortunately, another equivalence group present in *C. elegans* males provides us the opportunity to further understand the evolution of patterning networks and the molecular nature of these networks. Previous work has suggested that Wnt signaling, which functions in both *C. elegans* and *P. pacificus* vulval development, may also be involved in the specification of the male hook competence group (HCG), which has similar developmental origins to the VPCs (Sternberg and Horvitz, 1988).

In *C. elegans* males, the posterior Pn.p cells, P9.p, P10.p and P11.p, form the HCG (Sulston and White, 1980). Cell lineage studies and electron microscopic reconstruction by Sulston et al. (1980) demonstrate that the P10.p lineage generates the major components of the hook sensillum, including a hook structural cell, two supporting cells (hook socket cell and sheath cell), and two hook sensory neurons (HOA and HOB; Fig. 1A). The hook sensillum is a male copulatory structure involved in vulva location behavior during mating (Liu and Sternberg, 1995; Sulston et al., 1980). If P11.p or P10.p is killed using laser microsurgery, the adjacent anterior Pn.p (P10.p or P9.p) can substitute for the missing posterior cell. This posterior-to-anterior direction of recruitment after cell killing designates P11.p as primary (1°), P10.p as secondary (2°), and P9.p as tertiary (3°), so wild-type male P(9-11).p cells exhibit an invariant fate pattern of 3°-2°-1°. Each HCG cell fate has a distinct cell division pattern and produces different types of descendants (Fig. 1A-C).

The VPC and HCG equivalence groups not only have similar developmental origins and choices of three potential fates but also both require LIN-12/Notch to specify the 2° fate (Ferguson et al., 1987; Greenwald et al., 1983; Sternberg and Horvitz, 1989). Furthermore, similar to vulval development, LIN-12 appears to inhibit adjacent 1° HCG fates: in one of twelve *lin-12(null)* males, both P10.p and P11.p expressed the 1° fate; in the remaining eleven, P10.p was 3° (Greenwald et al., 1983). In addition, only the cells expressing the 1° and 2° fates of each equivalence group generate progeny that are required for the structure or function of the tissue (Sternberg and Horvitz, 1986; Sulston and White, 1980).

Since we discuss the effects of Wnt and EGF signaling on HCG specification, it is important to note that both signaling pathways can influence the size of the HCG due to an earlier role in development. Prior to HCG specification, the parent of P11.p, P11, is a member of the P11/12 equivalence group. Mutations in components of the EGF and Wnt signaling pathway affect P12 specification and thereby

alter the number of cells in the HCG (Jiang and Sternberg, 1998). Reduced EGF or Wnt signaling can cause a P12-to-P11 transformation in cell fates, thus adding an extra cell, P12.p, into the HCG and generating a spatial pattern of 3°-3°-2°-1° among P(9-12).p. Conversely, increased EGF signaling (e.g., in *lin-15(null)* mutants) causes a P11-to-P12 transformation, thereby reducing the HCG to only two cells (P9.p and P10.p). Because these effects on P11/P12 specification are incompletely penetrant, it is still possible to study the effects of these mutations on the patterning of a HCG of normal size.

In this study, we first characterize each HCG fate. Next, we demonstrate that the presence of the 1° fate is required for specification of the 2° fate and provide evidence that HCG induction occurs during or prior to the mid-L2 stage. We subsequently analyze the roles of EGF and Wnt signaling during hook patterning and provide evidence that Wnt and EGF pathways cooperate to promote the 1° HCG fate. Wnt signaling also acts during execution of the 1° fate as well as 2° fate specification and execution.

Materials and methods

General methods, nomenclature and strains

C. elegans strains were cultured at 20˚C according to standard procedures (Brenner, 1974). The alleles and transgenes used in this work are listed in Table S7. The strains used in this work are listed in Table S7. The *him-5* allele *e1490* was used to obtain males except for cases where the mutation of interest was linked to *him-5*, in which case *him-8* was used (Hodgkin et al., 1979).

HS::CAM-1

To reduce the level of Wnts, an extrachromosomal HS::CAM-1 transgene, *syEx710*, was used (Green et al., 2008). 20 to 24 hours after heat-shock, HCG lineages were followed in HS::CAM-1 and HS::CAM-1; *lin-44(n1792lf)* males starting from the mid-L3 stage.

lin-17::GFP expression

To examine *lin-17*::GFP expression, we crossed *syEx676(lin-17*::GFP*)* hermaphrodites with *him-5(e1490)* or *him-8(e1489)* males to yield F1 males carrying the extrachromosomal array. There was no difference in *lin-17*::GFP expression between *him-5(e1490)/+* and *him-8(e1489)/+* males.

Microscopy

Cell anatomy and lineages were examined in living animals using Nomarski differential interference contrast optics as described (Sulston and Horvitz, 1977). A Chroma Technology High Q GFP long pass filter set [450 nm excitation, 505 nm emission] was used for viewing both GFP expression and autofluorescence. Cells were killed in larvae with a laser microbeam as previously described, and the recovered animals were inspected for HCG patterning and marker expression (Avery and Horvitz, 1987; Sulston and White, 1980).

RNAi

The *lin-3* RNAi clone F36H1.4 was from the OpenBiosystems library; a feeding protocol similar to that previously described was used with minor adaptations (Kamath et al., 2001): after transferring 3 young adult hermaphrodites onto each RNAi plate, we incubated them at 22°C and did not remove them from the plates.

Results

Biology of the male hook competence group (HCG)

A description of the behavior of the male HCG cells is required to understand the experiments described in this work. Prior to the L3 stage, the distance between the nuclei of P9.p and P10.p is almost equal to the distance between P10.p and P11.p (Fig. 1D). During the early-to-middle L3 stage, P10.p and P11.p move to the posterior and closer to each other until just before the first round of HCG divisions (Fig. 1E). P9.p, which rarely migrates or divides, will occasionally migrate posteriorly and divide once to produce two cells that join hyp7. Sulston and Horvitz (1977) observed that 4 of 17 P9.p cells divided. Therefore, the 3° fate is to fuse with hyp7, sometimes dividing first. P10.p and P11.p divide multiple rounds during the mid-to-late L3 stage, the same time at which the VPCs divide in hermaphrodites. After the completion of cell divisions by the L3 lethargus, all nine P10.p descendants and the three posterior-most P11.p descendants align longitudinally at the ventral midline (Fig. 1B and 5A). The three posterior P11.p descendants are epidermal cells associated with the hook sensillum and form a spot of sclerotized cuticle (with autofluorescence) at the cloaca of adult males (Sulston et al., 1980). The four anterior offspring of P11.p are in slightly lateral positions and become preanal ganglion neurons. During the L4 stage, the hook structural cell, P10.papp, migrates posteriorly and forms an invagination (with the three posterior-most P11.p descendants) just anterior to the anus (Fig. 1F). P10.papp also forms the characteristic anchor-like structure within the invagination. In adults, the hook is an arrowhead-shaped sclerotic structure with autofluorescence (Fig. 1G).

Molecular markers of hook fates

We used three transcriptional GFP reporters as markers of HCG lineages. *eat-4* encodes a glutamate transporter (Bellocchio et al., 2000; Lee et al., 1999). We identified PVV (P11.paaa), based on both its position and cell-killing experiments, as the only neuron expressing *eat-4*::GFP beginning in the late L4 stage and continuing throughout adulthood (Fig. 1H-I). The cilium structural gene *osm-6* is expressed in both HOA (P10.ppa) and HOB (P10.ppap), and the homeobox gene *ceh-26* is expressed in HOB (Collet et al., 1998; Yu et al., 2003) (Fig. 1J-K). Therefore, *eat-4*::GFP is a 1° lineage marker, while *ceh-26*::GFP and *osm-6*::GFP are 2° lineage markers.

To determine the mechanism of HCG patterning and to identify pathways involved in this process, we utilized lineage analyses, hook structural cell features, laser microsurgery, and lineage-specific gene expression to examine 1° and 2° HCG cell fate specification and execution.

2° fate specification depends on the presence of a 1°-fated cell

Several observations suggested that the presence of a 1°-fated cell is required for specification of the 2° fate. First, an isolated P9.p adopted either a 1° or 3° fate but never a bona fide 2° fate: when both P10.p and P11.p (or the parents of P10.p and P11.p) were killed, P9.p adopted a normal 3° fate in 25 animals, a 1° fate in two animals, and an abnormal fate in six animals (see Table 1 legend for details). Second, in males in which P11.p was killed, P10.p did not adopt the 2° fate but instead adopted the 1° fate, and P9.p often adopted the 2° fate (Table 1). The failure of P9.p to consistently adopt the 2° fate in this situation might be a consequence of a delay in adoption of the 1° fate by P10.p, which would in turn reduce the efficiency of 2° fate formation by P9.p. Third, in the majority of males in which P10.p was killed, P9.p migrated posteriorly next to P11.p and acquired the 2° fate (Table 1). In two males in which P10.p was killed, P9.p did not migrate next to P11.p and adopted a 3° fate (Table 1), suggesting that proximity to a 1°-fated cell is required for specification of the 2° fate.

The LIN-12/NOTCH pathway appears to mediate the interaction between 1° and 2° cells since *lin-12(lf)* males are hookless as a result of deficient 2° fate formation (Greenwald et al., 1983). Conversely, abnormal activation of the LIN-12 pathway releases the dependence of 2° fate specification on a proximal 1° cell fate in the HCG. A *lin-12(gf)* mutation enables all three cells of the HCG to each adopt a 2° fate, generating up to three hook sensilla. Using the *osm-6::gfp* hook neuron marker, we found that *lin-12(n137gf)/lin-12(n676n909lf)* mutants generated extra pairs of hook neurons associated with each ectopic hook (Fig. S1). No PVV expression of *eat-4::gfp* was detected in *lin-12(n137gf)/lin-12(n676n909lf)* animals with three hooks, suggesting that the 2° fates are generated in the absence of a 1°-fated cell (n=59). Therefore, our data support previous findings that LIN-12 signaling is not only necessary but sufficient for 2° fate specification.

Time of HCG Specification

To investigate when HCG fates are determined, we killed individual members of the HCG at various times. Fate replacements after cell killing revealed that the mid-L2 stage (approximately 20 hours after hatching) is the latest time point at which an adjacent anterior cell is able to substitute for a missing posterior fate within the HCG (Table S1). We found that when P11.p was killed later than the mid-L2 stage, P10.p never assumed the 1° fate and always adopted the 2° fate, suggesting that 2° fate specification occurs during or prior to the mid-L2 stage. Furthermore, 1° HCG specification probably

also occurs prior to the mid-L2 stage, since we found that 2° fate specification likely requires the presence of the 1° fate.

P9.p usually fuses with hyp7 some time after the late L1 stage, leading to a loss of its greater developmental potential (Sulston and Horvitz, 1977). To determine when P9.p can respond to patterning signals, we monitored the time of P9.p fusion by examining AJM-1–GFP expression. AJM-1–GFP is localized to apical junctions of epithelial cells and disappears when cells fuse (Gupta et al., 2003; Sharma-Kishore et al., 1999; Shemer et al., 2000). In addition to P(9-11).p, four central Pn.p cells, P(3-6).p, also remain unfused in males during the L1 stage (Kenyon, 1986; Wang et al., 1993). We found that AJM-1–GFP was expressed in P(3-6).p and P9.p until the mid-L2 stage (Fig. 2A-E). As non-HCG-fated P(3-6).p cells gradually lost AJM-1–GFP expression and fused with the hyp7 epidermis during the mid-to-late L2 stage, P9.p showed a similar cell fusion pattern (Fig. 2F-H): AJM-1–GFP was expressed in 4 of 9 mid-to-late L2 stage males and in only 2 of 12 early L3 males. In both L3 animals with AJM-1–GFP expression, P9.p was slightly posterior to its wild-type position, which probably corresponds to the situation in which P9.p divides once. Therefore, P9.p fuses with hyp7 during the mid-to-late L2 stage (consistent with our results regarding the time of cell-fate commitment) and appears to be unable to substitute for a missing 2° cell after this time.

EGF signaling is sufficient but might not be necessary for 1° fate specification

Since LIN-3/EGF is the major inductive signal during vulval development and is expressed in the male blast cells, U and F (Hwang and Sternberg, 2004), we tested whether EGF signaling induces hook development. In hermaphrodites, *lin-15(null)* mutations cause increased EGF signaling due to the production of ectopic LIN-3/EGF (Clark et al., 1994; Cui et al., 2006; Huang et al., 1994). It is not known if *lin-15* mutations cause ectopic LIN-3/EGF in the male. However, we observed that *lin-15(null)* males exhibit an ectopic hook phenotype that is completely suppressed by *sy97*, a severe reduction-of-function allele of *let-23/EGFR*, indicating that the effects of *lin-15* are mediated through *let-23/EGFR* during hook development in the male (data not shown). To analyze the effects of *lin-15* on hook development, we followed the lineages of *lin-15(null)* males and found that P9.p in two of seven mutants generated a 1°-like lineage, suggesting that EGF signaling can promote the 1° fate (Table 1, Fig. S2). Furthermore, in *lin-15(null)* males in which P10.p was killed and the P10.p debris separated P9.p and P11.p (presumably blocking the lateral 2° signal produced by P11.p from reaching P9.p), both P9.p and P11.p adopted the 1° fate (Table 1). In most *lin-15* mutants in which P10.p was

absent, however, P9.p migrated next to P11.p and adopted the 2° fate. Therefore, to determine if increased EGF signaling is sufficient to promote the 1° fate in all HCG cells, we examined *lin-15(null)* animals in which LIN-12/NOTCH signaling is absent. In *lin-12(n137 n720null)* animals, P10.p never adopts the 2° fate and adopts the 3° fate in most cases (Greenwald et al., 1983). Lineage analysis of two *lin-12(n137 n720null); lin-15(n309null)* males showed that P9.p, P10.p and P11.p each generated a 1° lineage (Table 1). Therefore, in the absence of the lateral signal mediated by LIN-12/Notch, increased EGFR-RAS signaling is sufficient to induce P9.p and P10.p to adopt the 1° fate.

If EGF signaling is sufficient to specify the 1° fate, and the 1° cell signals laterally to specify the 2° fate, we would expect to see ectopic 2° fates caused by a 3°-to-2° fate transformation in *lin-15* mutants or other mutants in which there is excessive EGF signaling. Indeed, we observed that in 4 of 7 *lin-15(null)* males, P9.p adopted a non-tertiary fate with 2° characteristics: in two of these animals, P9.p adopted the 2° fate and in the other two, P9.p generated a 2°-like lineage (Table 1, Fig. S2). However, the 2° fate transformation of P9.p in *lin-15(null)* mutants was not complete, since an extra hook neuron was never detected in *lin-15(null); osm-6*::*GFP* males that had two hooks (Fig. 3, Table S2). A similar result was obtained using the *ceh-26*::GFP marker (Table S2). Although gain-of-function (gf) mutations in *let-23/EGFR* or *let-60/Ras* did not cause ectopic 2° HCG fates in males (data not shown), *let-23(gf); let-60(gf)* double mutant males showed abnormal P9.p specification similar to *lin-15* mutants. We found that in 4 of 8 *let-23(sa62gf); let-60(n1046gf)* males, P9.p divided more than once prior to the L4 stage. These eight males were subsequently examined in the late L4 or adult stage, and two had an anterior hook-like invagination or an autofluorescent protrusion (in addition to the P10.p hook), indicating that P9.p had generated a 2° or 2°-like lineage.

Although severe reduction-of-function mutations in EGF pathway components, such as *let-23(sy97)* and *sem-5(n1619)*, can cause a vulvaless phenotype in hermaphrodites (Aroian and Sternberg, 1991; Aroian et al., 1990; Chamberlin and Sternberg, 1994), they did not cause HCG patterning defects in males carrying those same mutations: all 14 *let-23(sy97)* and all 7 *sem-5(n1619)* males scored had wild-type hook lineages. The early larval lethality caused by null alleles of *lin-3/EGF, let-23/EGFR, sem-5/Grb-2, let-60/Ras* and *mpk-1/MAPK* preclude their use for studying the requirement of EGF signaling in HCG specification. Therefore, we examined *lin-3* RNAi-treated males and found them to have no hook lineage defects (Fig. 4B, Table S5). We cannot rule out that EGF signaling is necessary for HCG fate specification because RNAi might compromise gene activity only partly in our assay.

lin-3 RNAi can abolish vulval induction in hermaphrodites but the vulval defects are more penetrant in animals which are sensitized to the effects of RNAi (C. Van Buskirk, personal communication).

Wnt signaling is required for 1° and 2° HCG fate specification and execution of the 1° fate

Unlike the VPCs in which EGF signaling is necessary and sufficient for fate specification, we have shown that EGF signaling can specify a 1° hook fate but that severe reduction-of-function (rf) mutations in this pathway have no detectable effects on 1° fate specification. Therefore, another signaling pathway is likely to play a role in this process. We examined the role of Wnt signaling in hook development, because P10.p and P11.p *lin-17/Frizzled* mutants have been reported to generate an abnormal number of descendants and not divide in some cases (Sternberg and Horvitz, 1988). In addition, *C. elegans* has five Wnt-like genes (Korswagen et al., 2002): *egl-20*, *lin-44*, *mom-2*, *cwn-1* and *cwn-2*, each of which is expressed in some cell of the male tail. *egl-20* has been reported to be expressed in the anal depressor muscle and in the male blast cells P9/10, K, U, F and B in the tail (Whangbo and Kenyon, 1999). In hermaphrodites, *lin-44* is expressed in the tail hypodermis (Gleason et al., 2006; Herman et al., 1995; Whangbo and Kenyon, 1999), and we observe similar expression in males carrying a *lin-44*::GFP extrachromosomal reporter, *syEx670* (data not shown). We examined animals carrying *syEx556* (*cwn-1*::GFP), *syEx631* (*cwn-2*::GFP) and *syEx566* (*cwn-2*::GFP) extrachromosomal arrays and found that *cwn-1* was expressed in two cells dorsal to P11.p (likely DP6 and DA8), the diagonal muscles, the anal depressor muscle and cells in the ventral cord, while *cwn-2*::GFP was observed in some rectal gland cells (data not shown). Finally, we observed *mom-2* expression in the male blast cells B, F, Y as well as P12.p, T.a, T.p, hyp7, hyp8 and hyp10 in *syEx664* males (data not shown).

We found no defect in hook lineages of *egl-20(lf)* and *cwn-2(lf)* single mutants (Table S3) and the hook morphology of *mom-2(rf)* mutants was normal. *cwn-1(lf)* mutants also probably have wild-type hooks (discussed in the next paragraph). Only *lin-44(lf)* mutants had mild hook defects: 1° and 2° fate execution in *lin-44(n1792)* and *lin-44(n2111)* males were slightly aberrant (Fig. 4A, C, Table S3) and P11.p and P10.p never adopted the 3° fate in these animals. *lin-44* has previously been shown to be required for the polarity of certain asymmetric cell divisions in *C. elegans* (Herman and Horvitz, 1994). Indeed, we observed 2 of 12 *lin-44(n1792lf)* animals exhibited a defect in P11.pp polarity (Table S3). Furthermore, in about a quarter of *lin-44(lf)* mutants, P11.p generated eight cells instead of the wild-type number of seven progeny: P11.pa acquired P11.pp characteristics and instead of dividing

obliquely and producing descendants that adopted a neuronal fate as seen in the wild type, it divided in an anterior-posterior pattern and generated epidermal cells. However, P11.pa produced four granddaughter cells, the same number of offspring as wild-type P11.pa. P11.pp in these animals divided in a similar manner to P11.pa. Our observations suggest that *lin-44* acts during 1° and 2° fate execution and may be required to maintain the polarity of certain divisions within the P11.p lineage.

Since functional redundancy of the Wnt ligands has been demonstrated in other developmental events, we next constructed several Wnt double mutant strains (Gleason et al., 2006; Green et al., 2008; Inoue et al., 2004). We found that *cwn-1(lf)*; *cwn-2(lf)* and *cwn-1(lf)*; *egl-20(lf)* double mutants had wild-type hook lineages suggesting that the *cwn-1(lf)* single mutant has no hook defect (Table S3). Our results also suggested that *lin-44* and *egl-20* act together during 1° fate execution as well as to specify the 2° HCG fate: in all four *lin-44(lf)*; *egl-20(lf)* double mutants whose cell lineages were followed, P11.p did not divide in a wild-type manner and P10.p adopted a 3° fate (Fig. 4A, 4C, Table S3). Although the requirements of *lin-44* and *egl-20* for 2° fate specification may be indirect since the 1° fate is required to specify the 2° fate, we provide evidence later that Wnt signaling most likely acts directly to specify 2° fates in addition to influencing the 2° fate through its effects on the 1° fate (see section "The LIN-17/Frizzled Wnt receptor is required for 1° and 2° HCG fate specification").

However, the majority of *lin-44(lf)*; *egl-20(lf)* double mutants had a P12-to-P11 transformation and there were very few animals with a normal-sized HCG. Therefore, to reduce Wnt activity after P12 specification, we utilized a HS::CAM-1 transgene with the heat-shock promoter fused to the *cam-1* coding region (Green et al., 2008). CAM-1 is the sole ROR (Receptor tyrosine kinase-like Orphan Receptor) family member in *C. elegans* and has been demonstrated to sequester Wnts and to bind EGL-20, CWN-1 and MOM-2 *in vitro* (Green et al., 2007). When animals that carry the HS::CAM-1 transgene are heat-shocked, overexpression of the CAM-1 protein is expected to reduce the levels of EGL-20, CWN-1 and MOM-2. Although it is conceivable that ectopic CAM-1 activity in the HCG may influence hook specification in addition to sequestering the Wnt ligands, HS::CAM-1 animals that were subjected to either a 45 minute or 2 hour heat-shock (during the early L1 stage prior to hook induction) had wild-type hook lineages, suggesting that HS::CAM-1 is not sufficient to affect hook specification on its own. To further reduce the level of Wnts, we repeated the experiments in a *lin-44(lf)* background since CAM-1 does not bind LIN-44 *in vitro*. We found that P10.p adopted the 3° fate in about 40% of *lin-44(lf)*; HS::CAM-1 animals (heat-shocked for 45 minutes or 2 hours) and

P11.p adopted the 3° fate in 2 of 32 *lin-44(lf)*; HS::CAM-1 animals (heat-shocked for 2 hours) (Fig. 4A and C). Our results indicate that the HS::CAM-1 construct only influences hook specification in the absence of *lin-44*. Since we found that HS::CAM-1; *lin-44(lf)* animals that were heat-shocked prior to induction have a similar P10.p defect as *lin-44(lf)*; *egl-20(lf)* double mutants and CAM-1 does not appear to bind LIN-44 in vitro, our results agree with a role for CAM-1 in lowering Wnt levels (most likely EGL-20) cell non-autonomously rather than to cause ectopic signaling or disrupt signaling cell autonomously. Thus, our results suggest that Wnts are required for 1° and 2° HCG specification as well as 1° fate execution. As P11.p adopted the 3° fate only in *lin-44(lf)*; HS::CAM-1 animals that had been heat-shocked for 2 hours (Fig. 4A), it appears that the P10.p lineage is more sensitive to reduced levels of Wnt than the P11.p lineage.

The LIN-17/Frizzled Wnt receptor is required for 1° and 2° HCG fate specification and execution

1° HCG fate specification and execution

To examine the role of *lin-17* in hook development, we used the *n671* and *n677* null alleles (Sawa et al., 1996). We found that of 26 *lin-17(null)* males, P11.p failed to divide in one male and in five animals divided only once (Fig. 5B, Table S4). In these six *lin-17(lf)* animals, P11.p behaved like a wild-type P9.p, adopting the 3° fate (Fig. 4B and 5B). The 1°-to-3° fate transformation of P11.p in *lin-17* mutants indicates that LIN-17 plays a role in specifying the 1° fate in the hook.

Apart from its role in 1° fate specification, LIN-17 also functions during 1° fate execution. Of the 20 *lin-17(n671)* P11.p lineages we observed, P11.p in 12 males generated seven or eight descendants, close to the 7 descendants generated by wild-type lineages (Fig. 4B, Table S4). In the remaining three males, P11.p gave rise to fewer than seven descendants but did not acquire a 3° fate. A similar defect in P11.p specification was seen in *lin-17(n677)* mutants (Fig. 4B, Table S4). It has been suggested previously that *lin-17* might function in each cell division to maintain correct cell polarity (Herman and Horvitz, 1994; Sawa et al., 1996; Sternberg and Horvitz, 1988). In *lin-17(lf)* mutants in which P11.p generated eight cells, each P11.p daughter produced four granddaughter cells (in the same manner as we described for *lin-44(lf)* mutants), consistent with the hypothesis that LIN-17 is not just required to maintain the polarity of P11.p during the first division but also in later divisions. However, another possibility is that the 1° lineage defects of *lin-17(lf)* mutants are due to a defect in P11.p polarity resulting in two daughters that have hybrid fates. In addition, consistent with the lineage

analysis, we found that 89% of *lin-17*(*n671)* males lacked 1° PVV expression of *eat-4*::GFP (Table 2) which showed that P11.p descendants adopted an epidermal fate. The remaining 11% usually had two to five instead of one *eat-4*::GFP-expressing cell, and those cells were often located posterior to the normal PVV position (Fig. 5C-D), indicating that two or more P11.p descendants had adopted the same neuronal fate. Therefore, in *lin-17(lf)* males in which P11.p acquired a non-3° fate, P11.p descendants appeared either to fail to express individual identities or to mimic the cell fate of one another. Our results suggest that *lin-17* is required not only to specify the 1° fate but also functions during the differentiation of the 1° lineage descendants.

2° HCG fate specification and execution

Consistent with Wnts specifying the 2° HCG fate, we found that P10.p in *lin-17(lf)* mutants could generate a 3° fate or an abnormal lineage. In 9 of 47 mid-L3 *lin-17(n671)* males, AJM-1–GFP expression was absent in P10.p, indicating that P10.p had fused to hyp7 (data not shown). Second, lineage analysis revealed that in 14 of 20 *lin-17*(*n671)* males and 5 of 6 *lin-17(n677)* males, P10.p adopted the 3° fate as compared to 5 of 20 males in which P11.p adopted the 3° fate (Fig. 4B, 4C, Table S4). Third, about 90% of *lin-17(n671)* adults were hookless, and the remainder exhibited some degree of 2° fate differentiation and had a misshapen hook-like protrusion with autofluorescence at a position corresponding to P10.papp (the hook structure cell) before its posterior migration. Fourth, *ceh-26*::GFP and *osm-6*::GFP expression were absent in 100% and 95% of *lin-17(n671)* males, respectively (Table 2). Therefore, P10.p descendants in *lin-17* mutants differentiate incorrectly and fail to express wild-type 2° fates, and the P10.p lineage appears to be more sensitive in *lin-17(lf)* mutants than the P11.p lineage. Based on lineage analysis and expression of both 2° fate GFP markers, we did not observe any *lin-17(lf)* males in which P10.p polarity was reversed. Therefore, our results suggest that LIN-17 functions in 2° fate specification and execution.

However, it is not clear if the effects of *lin-17* on P10.p are direct or indirect since 2° fate specification requires the presence of a 1°-fated cell. The severe hookless phenotype of *lin-17* mutants might be due only to insufficient lateral signaling because of LIN-17 requirements during 1° fate specification or caused by a synergistic effect of insufficient lateral signals from an underinduced P11.p and decreased Wnt pathway activities in P10.p. To clarify if the P10.p lineage defect in *lin-17(lf)* animals is solely a result of insufficient lateral signaling, we tested whether the hookless phenotype of *lin-17(lf)* males could be rescued by a *lin-12(gf)* mutation, which is sufficient to specify the 2° fate in the absence of a

1° fate. Indeed, we found that a slightly greater proportion of *lin-17(lf); lin-12(gf)/lin-12(null)* males had a hook compared to *lin-17(lf)* single mutants (Table 2). Furthermore, in 14 of 25 L4 *lin-17(lf); lin-12(gf)/lin-12(null)* males, we found that P9.p had divided more than once (as opposed to remaining uninduced, as in *lin-17* single mutants (Table S4), and both P10.p and P11.p adopted non-3° fates in 17 of 25 *lin-17(lf); lin-12(gf)/lin-12(null)* males (Fig. 5E). Therefore, activated LIN-12 signaling was sufficient to cause P(9-11).p to adopt non-3° fates and promoted 2° hook formation in the absence of *lin-17* function. However, P10.p adopted the 2° fate and never the 3° fate in all of *lin-12(gf)/lin-12(null)* mutants compared to 3 of 25 *lin-17(lf); lin-12(gf)/lin-12(null)* males in which P10.p adopted the 3° fate, indicating that *lin-17* is required to specify the 2° lineage in addition to *lin-12* because the *lin-12(gf)* mutation is usually sufficient to specify a 2° fate.

In addition, 2° fate execution in *lin-17(lf); lin-12(gf)/lin-12(null)* double mutants was defective: *eat-4*::GFP and *osm-6*::GFP expression were similar in *lin-17(lf); lin-12(gf)/lin-12(null)* and *lin-17(lf)* males (Table 2). Also, more than 85% of *lin-12(gf)/lin-12(null)* animals had two or three hooks, and each hook was accompanied by extra hook neurons (Fig. S1). By contrast, very few double mutants had two hooks, and the majority remained hookless (Table 2). Thus, reduced signaling through *lin-17* suppressed the multi-hook phenotype of the *lin-12(gf)* mutation, while the *lin-12(gf)* mutation partially suppressed the hookless defect of *lin-17(lf)* mutants.

In short, similar to its role in 1° fate specification, LIN-17 specifies the 2° fate and is also required for 2° lineage execution.

***lin-17/Frizzled* and *bar-1/ β-catenin* are expressed in the HCG**

To determine if Wnt signaling is acting directly in the HCG or patterning the HCG indirectly by acting in non-HCG cells, we looked at the expression pattern of Wnt signaling components downstream of the Wnt ligand(s). Using a transcriptional *lin-17*::GFP reporter, we confirmed the results of Sawa et al. (1996) that *lin-17* is expressed in male P(10-11).p lineages. During the early L3 stage, *lin-17*::GFP was expressed predominantly in P11.p and was barely detectable in P10.p (Fig. 6A). No expression was detected in P9.p. Subsequently, descendants of both P10.p and P11.p expressed *lin-17*::GFP, with slightly higher levels in the P11.p descendants (Fig. 6B). The spatially graded expression of a Wnt receptor in the HCG might indicate a difference in competence to respond to a Wnt signal and/or a

differential response to a graded Wnt signal (if, for example, there is positive feedback on *lin-17* expression by previous Wnt signals).

β-catenins are downstream components in the Wnt pathway (Nelson and Nusse, 2004). Of the four *C. elegans* β-catenins (*bar-1*, *sys-1, wrm-1* and *hmp-2*), *bar-1* is involved in canonical Wnt signaling (Eisenmann, 2005). Therefore, to assess whether the canonical Wnt signaling pathway is activated in P10.p and P11.p, we analyzed subcellular localization of a translational BAR-1–GFP transgene, *gaIs45*, which rescues the *bar-1* mutant phenotype *in vivo* (Eisenmann et al., 1998). The expression of BAR-1–GFP is consistent with activated Wnt signaling that stabilizes cytoplasmic BAR-1, thereby allowing BAR-1 to interact with POP-1/TCF, translocate to the nucleus and regulate the transcription of target genes (Miller and Moon, 1996). BAR-1–GFP expression first appeared in P11.p in the late L1 stage (Fig. 6C-D). In the early-to-middle L2 stage, BAR-1–GFP accumulated in the cytoplasm of P11.p in a punctate pattern (Fig. 6E), presumably resulting from the stabilization of BAR-1 in response to increased Wnt signaling. The punctate GFP fluorescence in the cytoplasm of P11.p rapidly decreased during the mid-to-late L2 stage. By the mid-L3 stage, just before P11.p divides, BAR-1–GFP expression appeared to be brighter in the nucleus than in the cytoplasm (Fig. 6F). The switch of cytoplasmic-to-nuclear BAR-1–GFP accumulation is initiated in the mid-to-late L2 stage, coincident with the time window critical for the specification of HCG cell fates.

BAR-1–GFP expression was undetectable in P10.p prior to cell division but became visible in the nucleus of the posterior daughter, P10.pp, suggesting that Wnt signaling through BAR-1 likely acts during fate execution of some descendants of the P10.p lineage. Although we did not observe *lin-17*::GFP expression in P9.p, faint, mostly cytoplasmic expression of BAR-1–GFP was sometimes seen in P9.p up to the mid-L2 stage, just before P9.p fuses with hyp7.

Consistent with our hypothesis that BAR-1 activity responds to Wnt signaling during HCG specification, the expression of BAR-1–GFP in P11.p cells was disrupted in *lin-17(lf)* mutants. Faint uniform GFP expression was present in some late L1 and early L2 *lin-17(lf)* males; however, by the early L3 stage, there was no detectable BAR-1–GFP expression in P11.p (Fig. 6G). Lack of expression might be caused by BAR-1 degradation in *lin-17* mutants, since activated Wnt signaling is required to stabilize β-catenin protein (Nelson and Nusse, 2004). The failure to establish nuclear BAR-1 expression by the L3 stage in *lin-17(lf)* mutants could be a sign of a failure to specify the 1°

HCG fate in P11.p. However, we were unable to study the requirements for *bar-1* in a HCG of normal size because 99% of *bar-1(lf)* animals have a P12-to-P11 transformation (Howard and Sundaram, 2002). Even though *bar-1(lf)* males do not have a normal sized HCG, we found that only 14% of *bar-1(ga80)* males lacked both a hook structure and hook neurons, and 30% had a partial 2° lineage defect, with either the hook structure or a hook neuron absent (n=71). Since the 1° fate is required to specify the 2° fate, the mild 2° lineage defects of *bar-1(lf)* mutants suggests that 1° fate specification in these animals is not severely affected. The low penetrance of hook defects caused by loss of *bar-1* activity, in comparison to the penetrance of *lin-17(lf)* mutants, indicates that other components of Wnt signaling downstream of LIN-17, such as other β-catenins (*hmp-2*, *sys-1* or *wrm-1*), are likely to be involved in HCG patterning (Herman, 2001; Kidd et al., 2005; Korswagen et al., 2000; Natarajan et al., 2001).

Reduction of EGF and Wnt signaling causes a synergistic decrease in HCG specification

Since we have shown that the Wnt signaling pathway plays a major role in HCG specification, perhaps acting partially redundantly with EGF signaling, we tested whether a decrease of Wnt signaling could reveal a requirement for EGF signaling. We therefore assessed the effects of *lin-3/EGF* RNAi in a *lin-17(lf)* background. All *lin-3* RNAi males examined had wild-type hook lineages, and *lin-17(n671)* males treated with the vector control L4440 RNAi displayed HCG lineage defects similar to *lin-17(n671)* males (Fig. 4B, Table S5, p=0.7759; Mann-Whitney U Test). However, reduced EGF signaling enhanced the *lin-17(n671)* 1° fate defect. In 12 of 19 *lin-17(n671); lin-3* RNAi males, P11.p adopted a 3° fate compared to only 4 of 20 *lin-17(n671);* L4440 RNAi males (Fig. 4B, Table S5, p= 0.0095; Fisher's Exact Test). Therefore, *lin-3* is important for 1° fate specification when LIN-17 activity is compromised.

To test further if a 1° fate is specified by the combined action of Wnt and EGF signaling, we determined whether increasing the activity of the EGF pathway could partially suppress the HCG defects caused by reduced Wnt signaling by examining HCG lineages in *lin-17(n671); let-60(n1046gf)* double mutants. As mentioned above, *let-60*(*n1046gf)* mutants have wild-type P10.p and P11.p lineages (Table S5). P11.p in all 20 *lin-17(n671); let-60(n1046gf)* males adopted a non-tertiary fate as compared to 15 of 20 *lin-17(n671)* males (Fig. 4B, Table S5, p=0.0471; Fisher's Exact Test), indicating that increased EGF signaling is able to suppress the 1°-3° fate transformation caused by a *lin-17(lf)* mutation. However, the P10.p and P11.p lineages of *lin-17(n671); let-60(n1046gf)* mutant

males were not completely wild-type: in 17 animals, P11.p and P10.p generated eight descendants, a phenotype seen in some *lin-17* single mutants (Table S5). This observation again points to a crucial role for LIN-17 in 1° and 2° fate execution and suggests that EGF signaling is sufficient for specification but not differentiation of the 1° and 2° lineages. Since the effects of EGF signaling on 2° fate specification may be due to it's effects on 1° fate specification, we conclude only that the EGF pathway acts together with LIN-17-mediated WNT signaling in specification of 1° HCG fates. The WNT pathway plays a major role and the requirement for EGF signaling is revealed only when Wnt signaling is compromised.

Discussion

In this paper, we have characterized signaling pathways that regulate male hook development in *C. elegans*. Our main conclusions are that Wnt and EGF signaling act together to specify the 1° lineage, while Wnt signaling is also required during 2° fate specification as well as execution of the 1° and 2° fate. Here, we summarize our results and compare hook development to vulval development in *C. elegans* and other species of nematodes.

Wnt and EGF signaling pathways are both involved in HCG development

Wnt signaling is required for 1° and 2° HCG fate specification and excecution

First, we propose that multiple Wnts contribute redundantly to 1° HCG specification. By observing lineages in heat-shocked *lin-44(n1792)*; HS::CAM-1 males (which are expected to have lower levels of Wnts) and *lin-17(null)* males, which lack a major Wnt receptor, we determined that Wnt signaling is a major signaling pathway involved in 1° HCG fate specification. Second, lineage analysis of *lin-44(n1792)*, *lin-44(n1792); egl-20(hu120)* and *lin-17(null)* males and the expression pattern of the *eat-4*::GFP 1° lineage-specific marker in *lin-17(null)* males indicated that Wnt signaling functions during 1° fate execution. Third, we provide evidence that *lin-17* is required to specify the 2° HCG fate since increased *lin-12/Notch* activity only partially rescues the defects in 2° HCG fates in a *lin-17* mutant. Fourth, by using 2° lineage-specific markers, we show that *lin-17* is necessary for differentiation of 2° lineage descendants. Previous cell-culture and *Drosophila* studies have suggested that Wnt and Notch signaling can act synergistically on the same cell (Couso et al., 1995; Espinosa et al., 2003). Wnt signaling might potentiate or be required for proper upregulation of Notch transcriptional targets during 2° fate specification in both the HCG and VPC equivalence groups. Consistent with our lineage analysis of Wnt pathway mutant males, LIN-17 and BAR-1/β-catenin are preferentially expressed in

P11.p (the presumptive 1° cell). In P11.p, the 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. The presence of BAR-1–GFP in P10.p descendants also agrees with our other results that Wnt signaling is required for 2° fate execution.

A role for EGF signaling during 1° HCG fate specification

We found that EGF signaling promotes a 1° HCG fate. However, the requirement for EGF signaling in 1° HCG fate specification is seen only when Wnt signaling activity is reduced. Decreased EGF signaling in an animal deficient in Wnt signaling has a synergistic effect on reducing 1° fate specification, but EGF signaling mutants have wild-type hook lineages. In addition, EGF signaling is sufficient to specify the 1° and 2° HCG fates when Wnt or Notch signaling is compromised: increased EGF signaling in the absence of 2° specification (i.e., in a *lin-12(null)* background) results in all cells in the HCG acquiring a 1° fate, while activation of EGF signaling suppresses the *lin-17* 1° and 2° HCG specification defect. We also found that hyperactivity of EGF signaling results in the adoption of a 2°-like fate by P9.p. One possible explanation is that the inductive signals, Wnt and EGF, are present posteriorly, closest to the 1° P11.p cell and furthest from P9.p, and thus the induced P9.p is biased to become a 2°-like cell by an induced 1° P11.p. Therefore, the role of the EGF pathway in 2° fate specification may be indirect, and we conclude only that EGF signaling is required for 1° fate specification. Although EGF signaling does not appear to be necessary for 1° fate specification, we cannot preclude a role for EGF signaling in specifying HCG lineages since there are no viable null alleles of EGF signaling pathway genes. The incomplete penetrance of the 1° lineage defect of *lin-17*; *lin-3* RNAi animals might be a consequence of the inefficiency of RNAi treatment or indicate the existence of a second Wnt receptor or a third inductive signal that acts in hook development.

Previous cell-killing experiments did not identify the source of the inductive signal for hook development (Chamberlin and Sternberg, 1993; Liu and Sternberg, 1995; Sulston and White, 1980) (M. Herman and H.R. Horvitz, unpublished observations). Perhaps a small amount of diffusible signal secreted from the source cell(s) before the cell is killed is sufficient for HCG patterning. Another possibility is that the signal might be secreted from a cell or cells that cannot be identified without killing the animal, e.g., the hyp7 syncytial hypodermis. A third possibility is that the signal is redundant, and the correct combination of cells secreting signals has not yet been discovered. Our work suggests that the last explanation is plausible, since multiple Wnts and the EGF signal are

required for HCG specification. Most likely, multiple Wnts signal redundantly through LIN-17 (and perhaps other Frizzled receptors) to pattern the HCG together with the EGF signaling pathway.

Logic of how the fates of multipotent precursor cells are specified

We show that the Wnt and EGF pathways act together to specify the 1° HCG fate and are responsible for inducing hook development, similar to their roles in vulval development. Another similarity is that *lin-17/Frizzled* plays a role during 1° and 2° fate execution in both hook and vulval development (Ferguson et al., 1987; Wang and Sternberg, 2000). One difference is that the relative importance of Wnt and EGF signaling is reversed in HCG and VPC specification. During vulval development, the EGF pathway is the major inductive pathway, while Wnt signaling appears to play a lesser role (Eisenmann et al., 1998; Gleason et al., 2006; Sternberg, 2005). In contrast, Wnt signaling is the major hook inductive pathway, whereas EGF signaling is less important and its role is seen only when Wnt signaling is compromised (Fig. 7).

EGF and Wnt signaling are thought to be required for two separate events at two different stages during vulval development. The current view is that maintaining VPC competency during the L2 (i.e. to prevent cell fusion to hyp7 otherwise known as the "F" fate) and induction during the L3 are separate events (Eisenmann et al., 1998; Myers and Greenwald, 2007). P4-8.p in hermaphrodites never adopt the F fate and are always induced, while P3.p adopts the F fate in 50% of hermaphrodites during the L2 and the 3° VPC fate in 50% of hermaphrodites during the L3. The Wnt pathway prevents fusion during the L2 stage, and reduced Wnt signaling often results in the generation by P5.p-P7.p of a 3° or F fate and in the generation by P3.p, P4.p and P8.p of a F fate. In addition, reduced EGF signaling enhances the F fate defect in a reduced Wnt signaling background. During hook development, P9.p resembles P3.p in hermaphrodites as it either fuses to hyp7 or divides once and fuses to hyp7. However, unlike P3.p in hermaphrodites, P9.p in the majority of males fuses during the mid-to-late L2 stage. Because the time of HCG induction determined by cell killing experiments is the mid-L2 stage or earlier, the maintenance of HCG competence (i.e. to prevent fusion to hyp7) and HCG induction do not appear to be temporally separate events. Furthermore, P11.p and P10.p are observed to fuse inappropriately with hyp7 in heat-shocked *lin-44(n1792)*; HS::CAM-1 males (which are expected to have lower levels of Wnts), suggesting that Wnt inductive signaling in the L2 prevents fusion of cells in the HCG in addition to inducing hook fates. Therefore, unlike vulval development, one signaling event in the L2 stage prevents fusion and induces hook development. Since the same

signals act to prevent fusion as well as promote induction in both VPC and HCG specification, our findings raise the possibility that maintaining competence and induction may not be separate events but the effects of the accumulation of a competence-promoting/inductive signal(s) over time. Cells that receive either no signal or too little signal will fuse (P3.p in the hermaphrodite and P9.p in the male). Cells that receive slightly more signal manage to overcome fusion during the L2 stage but do not receive enough to prevent exit from the cell cycle and fusion in the L3 after one round of division (P4.p and P8.p in the hermaphrodite). Cells that receive enough signal do not fuse and are induced to divide more than once (P5-7.p in the hermaphrodite and P10-11.p in the male).

Although the relative importance of the EGF and Wnt signaling pathways in VPC and HCG patterning differs, the same signal is utilized to specify the 2° fate in both equivalence groups. In vulval development, EGF acts through the EGF-receptor to cause the production of Notch ligands (DSL) in the cell closest to the source of the EGF, leading to Notch signaling in a neighboring cell (Chen and Greenwald, 2004). This relationship between EGF and Notch signaling has also been observed during *Drosophila* eye development (Tsuda et al., 2002). Preliminary data show DSL expression in P11.p (1°) during the time of HCG specification (A. Seah, unpublished observations), and it is likely that sequential signaling occurs to induce DSL expression and activate the Notch pathway in P10.p (2°). One possibility is that similar to vulva development, Notch lateral signaling in P10.p results from the upregulation of DSL ligand(s) in P11.p by EGF signaling. However, since Wnt signaling through LIN-17/Frizzled is the major patterning pathway in hook development, another possibility is that DSL ligand production in P11.p is controlled by Wnt signaling, instead of (or in addition to) EGF signaling. Several studies of mouse and *Drosophila* strongly suggest such a relationship between Wnt and Notch signaling. In particular, overexpression of Frizzled leads to transcriptional upregulation of a Notch ligand, Delta, in *Drosophila* (Fanto and Mlodzik, 1999), while reduced Wnt activity or a downstream component, Lef, results in lower levels of Delta in mice (Galceran et al., 2004; Nakaya et al., 2005). However the Notch ligand is produced, the Notch signaling pathway is probably used as a lateral signal since the DSL ligands act at a short range, consistent with our data that 2° HCG specification requires an adjacent 1°-fated cell.

The developmental history of a cell is important in its response to intercellular signals because of the factors available to interact with downstream components of the signaling pathway (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). When the Pn.p cells are generated in the L1, *lin-39* is expressed

in P(3-8).p while a different Hox gene, *mab-5*, is expressed in P(7-11).p, and both Hox genes are required to prevent fusion in the L1 stage (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993). It is not known how Hox gene expression is initiated in the Pn.p cells. The Wnt pathway prevents fusion by maintaining *lin-39/Hox* expression (which is first observed in the L1 stage), while the EGF pathway does not appear to affect *lin-39/Hox* at this time (see below) (Eisenmann et al., 1998; Wagmaister et al., 2006). One possibility is that different Hox genes may confer the specificity of response to the EGF and Wnt pathways in the VPCs and HCG. In addition to preventing fusion during the L2, *lin-39/Hox* is also upregulated in response to the EGF signal and required to specify vulval fates during the L3. Several observations suggest that *mab-5* acts to specify hook fates in males. First, excessive Notch signaling, which specifies both the 2° VPC and 2° HCG fates, in *lin-12(gf)* males causes P(3-8).p to acquire vulval fates and P(9-11).p to generate hook fates, implying that P(3-8).p and P(9-11).p have different tendencies to produce vulval and hook lineages, respectively (Greenwald et al., 1983). Second, overexpression of MAB-5 in *lin-39(rf)* hermaphrodites suggests that MAB-5 acts to specify hook versus vulval fates (Maloof and Kenyon, 1998). Further investigation into the role of *mab-5* during hook development will be necessary to understand how EGF, Wnt and Hox genes interact to specify distinct fates.

Evolution of the inductive signal

Although the patterning of the *C. elegans* hook and vulva share some similarities, hook patterning in *C. elegans* males might be more similar to vulval development in more ancestral nematode species. Recently, it was reported that *Ppa-egl-20/Wnt*, *Ppa-mom-2/Wnt* and *Ppa-lin-18/Ryk* in *P. pacificus* induce vulva development (Tian et al., 2008). EGF signaling does not appear to act in vulva development in *P. pacificus*, although it is possible that a role for the EGF pathway might be uncovered in Wnt signaling mutants as it has been for *C. elegans* hook development. Furthermore, studies of vulval development in some species, such as *Mesorhabditis*, were unable to identify a source of an inductive signal (Sommer and Sternberg, 1994) as has been the case for *C. elegans* hook development. Perhaps vulva development in those species also depends on Wnt signals from multiple sources. In this view, since the *Mesorhabditis* group is an outgroup to the diplogastrids (which includes *P. pacificus*) and *Caenorhabditis* group (Kiontke et al., 2007), the ancestral mode of epidermal fate specification would be through Wnts and their respective receptors, while the EGF induction of fates would be a more recently evolved character (Fig. 7).

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Figure legends

Figure 1. Development of the male hook sensillum competence group (HCG). (A) Cell division patterns of P(9-11).p, adapted from Sulston et al. (1980). so, socket cell; sh, sheath cell. Three-letter names refer to specific neurons. (B) HCG divisions during the L3 stage. Left lateral views. } indicates sister cells, L indicates left plane, R indicates right plane. (C) Mid-L2. Distances from P9.p to P10.p and from P10.p to P11.p are similar. (D) Early L3. P10.p and P11.p migrate posteriorly. (E) Late L4. P10.papp, the hook structure cell, formed an invagination (arrowhead) just anterior to the anus. HOA and HOB are hook neurons generated by the 2° P10.p lineage. (F) Adult sclerotic hook structure (arrowhead). (G, H) *eat-4*::GFP expression in PVV, a P11.p (1°) descendant. (I) *osm-6*::GFP in HOA and HOB. (J) *ceh-26*::GFP in HOB. For B-J: Left lateral views (anterior left, ventral down). Cell nucleus (arrows). Scale bar in I, 20 μm for C-J. (K) Arrangement of nuclei in the adult, adapted from Sulston et al., 1980. Ventral view.

Figure 2. P9.p fusion with hyp7 during the mid-to-late L2. In all panels showing GFP fluorescence, an unfused Pn.p cell expresses AJM-1–GFP (observed as a green line at the ventral side of the cell, toward the bottom of the figure). The junction of adjacent unfused Pn.p cells is marked by a bright dot (arrowhead). (A-C) Early L2. Unfused P(3-6).p (A) and P(9-11).p (B, C) with AJM-1–GFP expression. (D, E) Mid-L2. Unfused P9-11.p cells retained AJM-1–GFP expression. (F-H) Mid-late L2. AJM-1–GFP expression was observed in P10.p and P11.p (G, H) but absent in P(5-6).p (F) and P9.p. Left lateral views. Scale bar in A, 20 μm for A-H.

Figure 3. The *lin-15(e1763)* mutation causes a partial 2°-fate transformation of P9.p in males with wild-type P12 specification. (A-C) The P10.p-derived wild-type hook invagination (h-in) was accompanied by a pair of hook neurons HOA and HOB, expressing *osm-6::*GFP (n=23). However, the P9.p-derived ectopic hook invagination was not accompanied by a pair of neurons expressing *osm-6::*GFP. Left lateral views. Scale bar in C, 20 μm for A-C.

Figure 4. Wnt and EGF signaling cooperate during 1° HCG specification. (A) P11.p lineages in Wnt mutants. Our data suggests that of the five Wnt-like genes in *C. elegans*, only mutations in *lin-44* caused defects in 1° HCG specification. However, the P11.p proliferation defect of *lin-44(lf)* mutants was mild and P11.p always adopted a non-3° fate. When Wnt activity was further reduced in *lin-44(n1792)*; HS::CAM-1 animals (heat-shocked for 2 hours), P11.p adopted a 3° fate in 2 of 32 animals.

n, number of animals in which cell lineages were observed; 3°, cell did not divide or divided once (red); non-3°, cell generated 3-8 descendants (3-6 (yellow); wild-type 7 (light blue); 8 (green), more than 2 (gray)). (B) P11.p lineages in EGF or LIN-17/Frizzled Wnt Receptor mutants. P11.p in 25% of *lin-17(n671lf)* mutants adopts the 3° fate (wild-type males as compared to *lin-17(n671)* males, *p=0.0471, Fisher's Exact Test. Decreased EGF signaling by *lin-3* RNAi enhanced the 1° lineage defect of *lin-17(lf)* mutants and caused P11.p to adopt the 3° fate instead of a non-3° fate more frequently (*lin-17(n671)*; *lin-3* RNAi males as compared to *lin-17(n671)* males, **p=0.0095, Fisher's Exact Test); while increased EGF signaling by a *let-60(gf)* mutation prevented 3° fate transformation of P11.p in a *lin-17(lf)* background, causing P11.p to adopt an abnormal non-3° fate instead of a 3° fate (*lin-17(n671)*; *let-60(n1046)* males as compared to *lin-17(n671)* males, *p=0.0471, Fisher's Exact Test). Color scheme as in (A). (C) P10.p lineages in Wnt signaling mutants. P10.p in animals with lower levels of Wnt or that carried a *lin-17/Frizzled* null allele often adopted the 3° fate (wild-type males as compared to *lin-44(n1792)*; *egl-20(hu120)* males, ***p<0.0001, Fisher's Exact Test; HS::CAM-1 males heat-shocked for 45 minutes as compared to *lin-44(n1792)*; HS::CAM-1 males heat-shocked for 45 minutes, **p=0.0010, Fisher's Exact Test; HS::CAM-1 males heat-shocked for 45 minutes as compared to *lin-44(n1792)*; HS::CAM-1 males heat-shocked for 2 hours, **p=0.0003, Fisher's Exact Test; wild-type males as compared to *lin-17(n671)* males, ***p<0.0001, Fisher's Exact Test; wild-type males as compared to *lin-17(n671)* males, ***p<0.0001, Fisher's Exact Test. In addition, in *lin-17(lf)* males in which P10.p divided, P10.p generated an abnormal non-3° fate with 3-to-8 descendants. Color scheme as in (A), however, for non-3°, cell generated 3-7 descendants (yellow) and wild-type 9 descendants (dark blue).

Figure 5. Abnormal HCG lineages in *lin-17(lf)* males. (A) End of L3 lethargus in wild type, cell divisions of P10.p and P11.p were complete. (B) An early L4 *lin-17(n671)* male, just after the L3 molt, in which P11.p and P10.p adopted an uninduced 3° fate. P10.px refers to P10.pa and P10.pp. (C, D) A hookless *n671* adult with five *eat-4::GFP*-positive neurons (1°). (E) A L4 *lin-17(n671); lin-12(gf)/lin-12(null)* male in which P(9-11).p had proliferated in response to the activated LIN-12 pathway but the alignment of cells was abnormal, indicating a failure to differentiate correctly due to the lack of LIN-17 function. Left lateral views. Scale bar in A, 20 μm for A-E.

Figure 6. *lin-17*::GFP and BAR-1–GFP expression in the HCG. (A-B) Wild-type transcriptional *lin-17*::GFP expression (A1-2) Early L3. *lin-17*::GFP in P10.p was barely detectable but stronger in P11.p.

No expression was detected in P9.p. (B1-2) Mid-L3. P11.p descendants had brighter *lin-17*::GFP expression than P10.p descendants. Pn.px refers to Pn.pa and Pn.pp. (C-F) Wild-type dynamic BAR-1–GFP expression in P11.p. (C1-2) L1. Faint BAR-1–GFP expression observed in P12 daughters but not in the undivided P11. (D1-2) Late L1. Faint BAR-1–GFP expression observed in P11.p. (E1-2) Mid-L2. Bright cytoplasmic punctate GFP granules (small arrowheads) and faint nuclear GFP expression in P11.p. (F1-2) Mid-L3. BAR-1–GFP expression in P11.p became predominantly nuclear. (G1-2) Early L3 *lin-17(lf)* mutant. No BAR-1–GFP was observed in P11.p. Panels (D2), (F2) and (G2) were exposed for longer than images in the other panels. In fluorescence images, cells are outlined based on corresponding Nomarski images. P11.p (large arrow), P12.pp corpse (large arrowhead), other cells (small arrows). Left lateral views. Scale bar in A1, 20 μm for A-H.

Figure 7. Comparison of VPC and HCG patterning networks in *C. elegans* and *Pristionchus pacificus*. In the *C. elegans* hermaphrodite, the EGF signal is produced by the anchor cell and induces the 1° VPC fate. The Wnt pathway is required for VPC competence and has a minor role in induction. In the *C. elegans* male, the EGF and Wnt pathways participate in HCG specification. However, the relative contributions of these two pathways in hook development are likely different from their contributions in vulval development, as Wnt signaling plays a relatively major role in this process. In response to a high level of Wnt and EGF signal(s), the LIN-17 and LET-23 receptors, respectively, on the cell surface of P11.p activate downstream pathways to specify the 1° fate, which produces ligands (DSL) for LIN-12/Notch. In P10.p, activated LIN-12/Notch signaling by the adjacent 1° P11.p cell acts with a weak Wnt and/or EGF signal to promote the 2° HCG fate. P9.p receives little (if any) signal, and therefore usually fuses with hyp7, adopting a 3° fate. In *P. pacificus*, different Wnt ligands act to induce as well as inhibit vulval development. A lateral signal from P6.p induces P5.p and P7.p. to adopt the 2° fates. It is not known if this is mediated by LIN-12/Notch.

Table 1. Cell-cell interactions in the male HCG

Genotype	Cells killed (Stage)[a]	n[b]	Cell fates adopted		
			P9.p	P10.p	P11.p
Wild type	none	many[c]	3°	2°	1°
	P10 (early L1)	1[d]	2°	**X**	1°
	P10.p (mid-L1)	4	2°	**X**	1°
	P10.p (mid-L2)	7	2°	**X**	1°
	P10.p (mid-L2)	2	3°	**X**	1°
	P11 (early L1)	1	2°	1°	**X**
	P11.p (mid-L1)	5[d]	2°	1°	**X**
	P11.p (mid-L1)	1	3°	1°	**X**
	P11.p (late L1)	1[d]	3°	1°	**X**
	P11.p (mid-L2)	2	2°	1°	**X**
	P11.p (mid-L2)	2[d]	3°	1°	**X**
	P10, P11 (early L1)	6	3°	**X**	**X**
	P10, P11 (early L1)	1	ab[e]	**X**	**X**
	P10.p, P11 (mid-L1)	8[d]	3°	**X**	**X**
	P10.p, P11.p (mid-L1)	11	3°	**X**	**X**
	P10.p, P11.p (mid-L1)	5	ab[e]	**X**	**X**
	P10.p, P11.p (mid-L1)	2[d]	1°	**X**	**X**
lin-15(n309)[f]	none	1	3°	2°	1°
	none	2	2°-like[g]	2°	1°
	none	2	1°-like[h]	2°	1°
	none	2	2°	2°	1°
	P10.p (mid-L1)	6	2°	**X**	1°
	P10.p (L2)	4	2°	**X**	1°
	P10.p (L2)	2[i]	1°	**X**	1°
unc-32(e189) lin-12(n137 n720); lin-15(n309)	none	2	1°	1°	1°

[a] The larval stage (L1-L4) at which cell(s) were killed.

[b] Number of animals in which P(9-11).p cell lineages were examined.

[c] The cell fates of P(9-11).p have been determined in many unoperated wild-type males in this study and by others (Sulston and Horvitz, 1977; Sulston and White, 1980; Sulston et al., 1980).

[d] Similar results have been reported by Sulston and White (1980).

[e] The lineage was abnormal: in two animals, P9.p exhibited a reversed 1° fate in which the posterior daughter (P9.pp) adopted a wild-type P11.pa fate and the anterior daughter (P9.pa) adopted a wild-type P11.pp fate; in three animals, one P9.p daughter did not divide while the other divided to give three or more descendants; in one animal, both P9.p daughters gave rise to four daughters each.

[f] Because defective P12 fate specification in mutants deficient in the EGF signaling pathway at an earlier stage can cause defects in HCG specification at a later stage, we examined only mutants that had a wild-type P12 to study the effects of EGF signaling on HCG specification.

[g] P9.p divided in a 2°-like pattern and made a hook or hook-like structure (Fig. S2).

[h] P9.p divided in a 1°-like pattern and did not make a hook or hook-like structure (Fig. S2).

[i] In these animals, debris from the dead P10.p cell blocked P9.p from migrating next to P11.p.

X: this cell was killed by laser microsurgery.

Table 2. *lin-17* is required for 1° and 2° HCG fate execution

Genotype[a]	Marker expression (%)			2 or more hooks (%)	Hook at P(9-11).p (%)	% hookless	n[b]
	P9.p	P10.p	P11.p				
***eat-4*::GFP[c] expression (1°)**							
Wild type	0	0	100	0	100	0	117
lin-17	0	0	11	0	7	93	110
lin-12(gf)/lin-12(lf)[d,e]	0	0	56	86	100	0	138
lin-17; lin-12(gf)/lin-12(lf)[d]	0	0	9	6	33	67	94
***osm-6*::GFP[c] expression[f] (2°)**							
Wild type	0	100	0	0	100	0	many
lin-17	0	5	0	0	10	90	42
lin-12(gf)/lin-12(lf)	99	100	31	97	99	0	70
lin-17; lin-12(gf)/lin-12(lf)	3[g]	4[g]	0	1	21	79	134
***ceh-26*::GFP[c] expression (2°)**							
Wild type	0	100	0	0	100	0	many
lin-17	0	0	0	0	6	94	63

[a] The alleles used were: *lin-17(n671), lin-12(n137)* referred to as "*lin-12(gf),"* and *lin-12(n676n909)* referred to as "*lin-12(lf)"* (Greenwald et al., 1983). All strains contain *him-5(e1490)*.

[b] Number of animals scored.

[c] The integrated *eat-4*::GFP, *osm-6*::GFP, and *ceh-26*::GFP transgenes were *adIs1240, mnIs17,* and *chIs1200,* respectively (Table S6). The *eat-4*::GFP transgene *adIs1240* uses *lin-15(+)* as coinjection marker and strains bearing *adIs1240* might have had a *lin-15(n765)* mutation in the background.

[d] Animals examined carried at least one copy of the *lin-12(gf)* allele, and both strains also contained the transgene *mnIs17*, but *osm-6*::GFP expression was not scored.

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

[f] Animals were inspected at the late L4 stage for *osm-6*::GFP expression in HOA and HOB. Hook invaginations, instead of hooks, were scored in those males. However, *lin-17; lin-12(gf)/lin-12(lf)* mutant males were examined as adults because ectopic rudimentary hooks were more obvious in the adult than ectopic rudimentary invaginations in the L4. Identification of *osm-6*::GFP expression in P10.p versus P11.p descendants was determined by their relative anterior-posterior positions as well as the morphology and position of the associated hook invagination (Fig. S1).

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

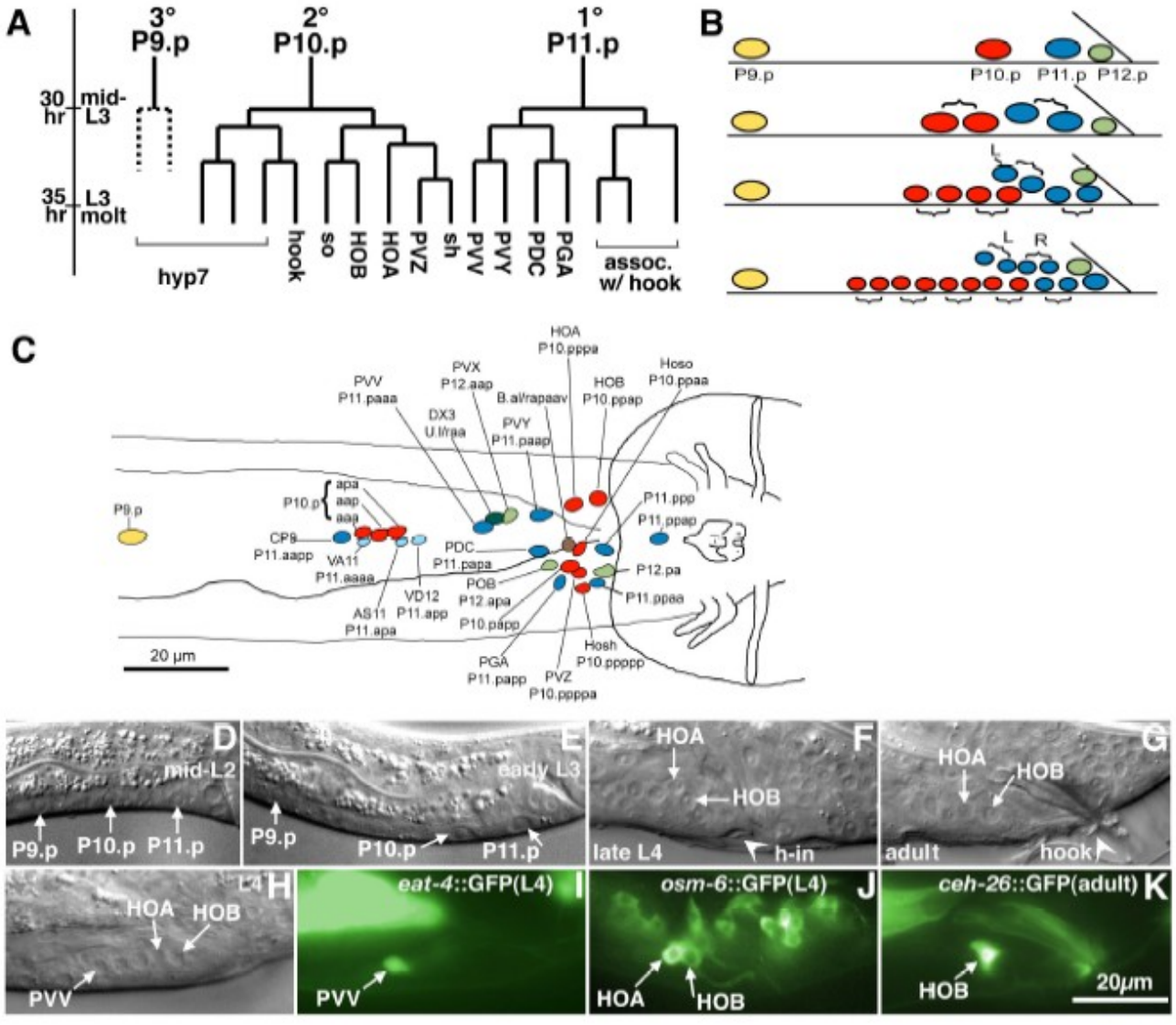


Fig. 1

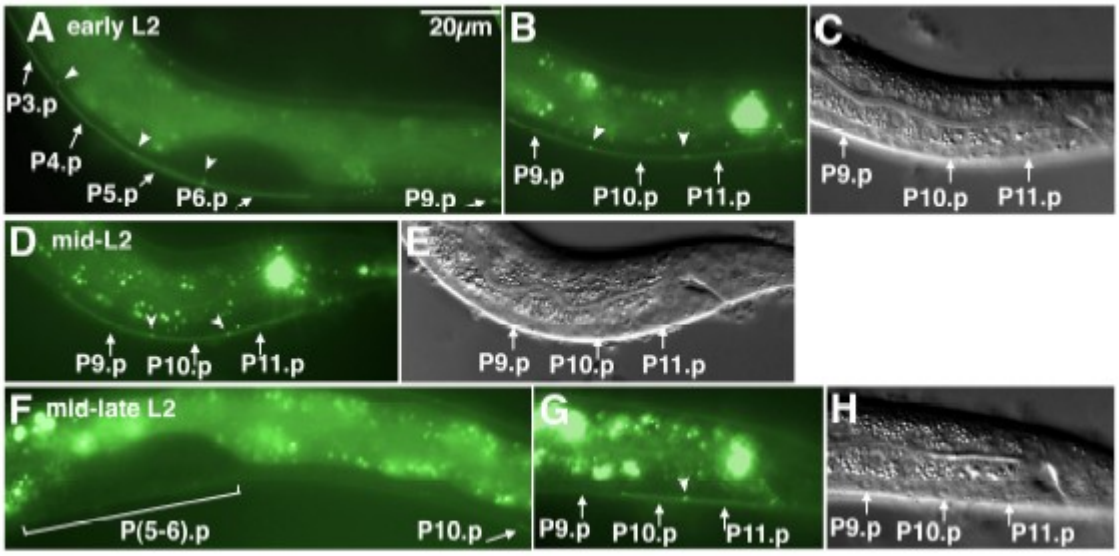


Fig. 2

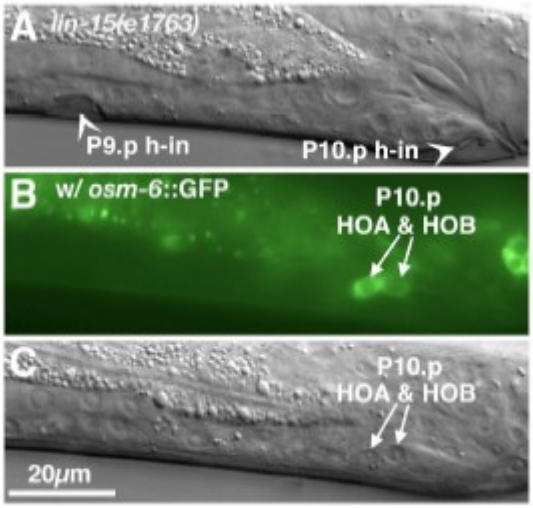
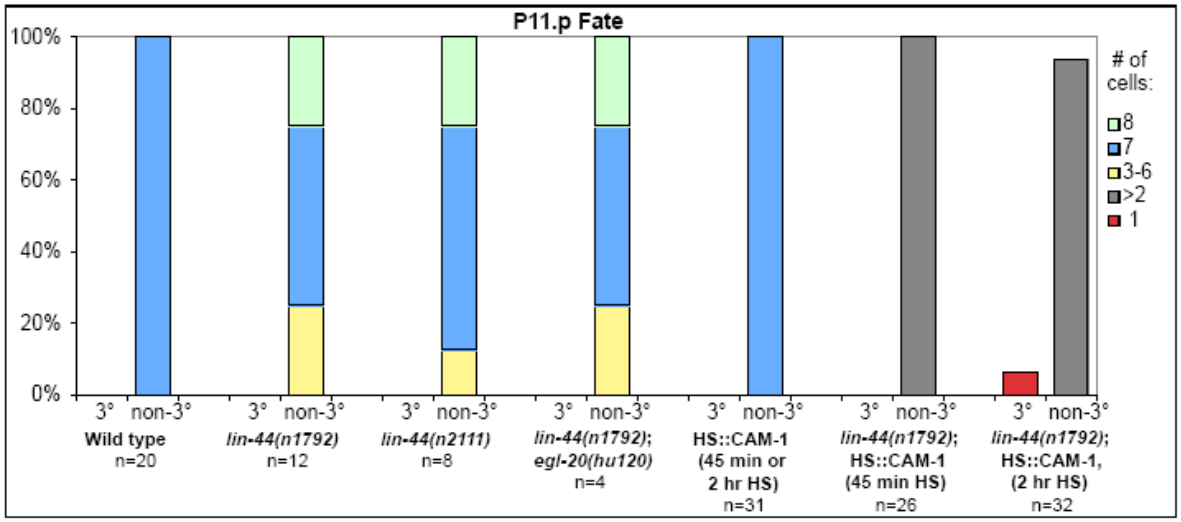


Fig. 3

Fig. 4A



4B

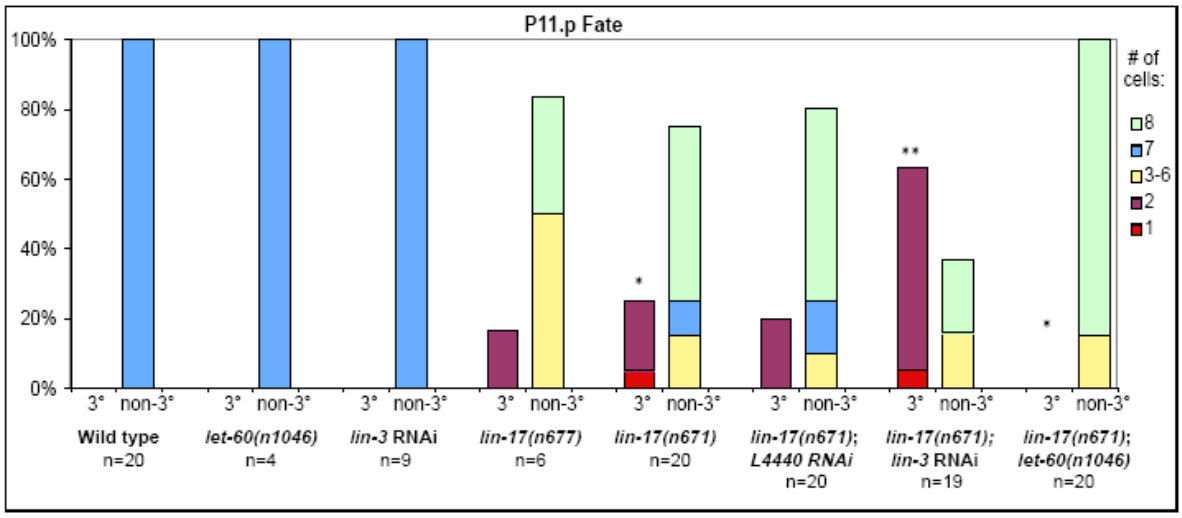
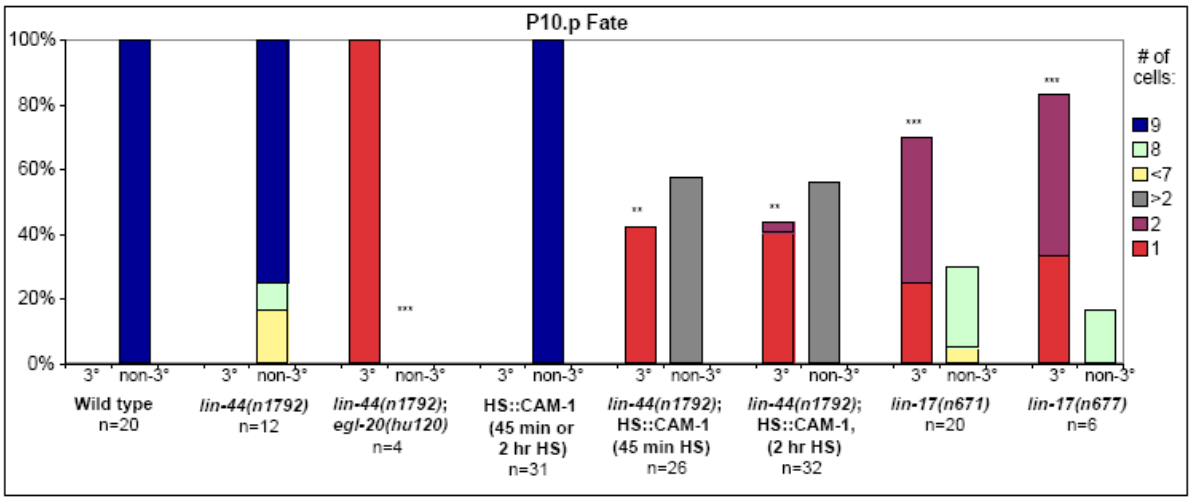


Fig. 4C



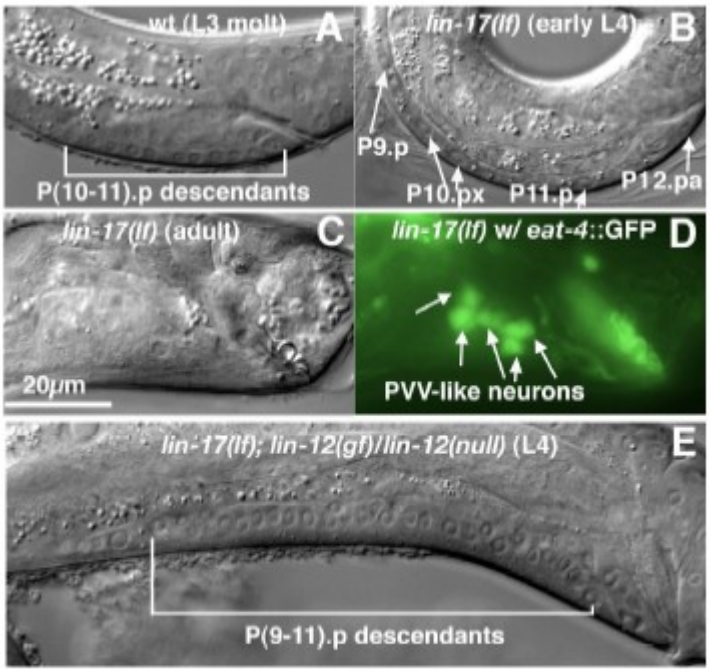


Fig. 5

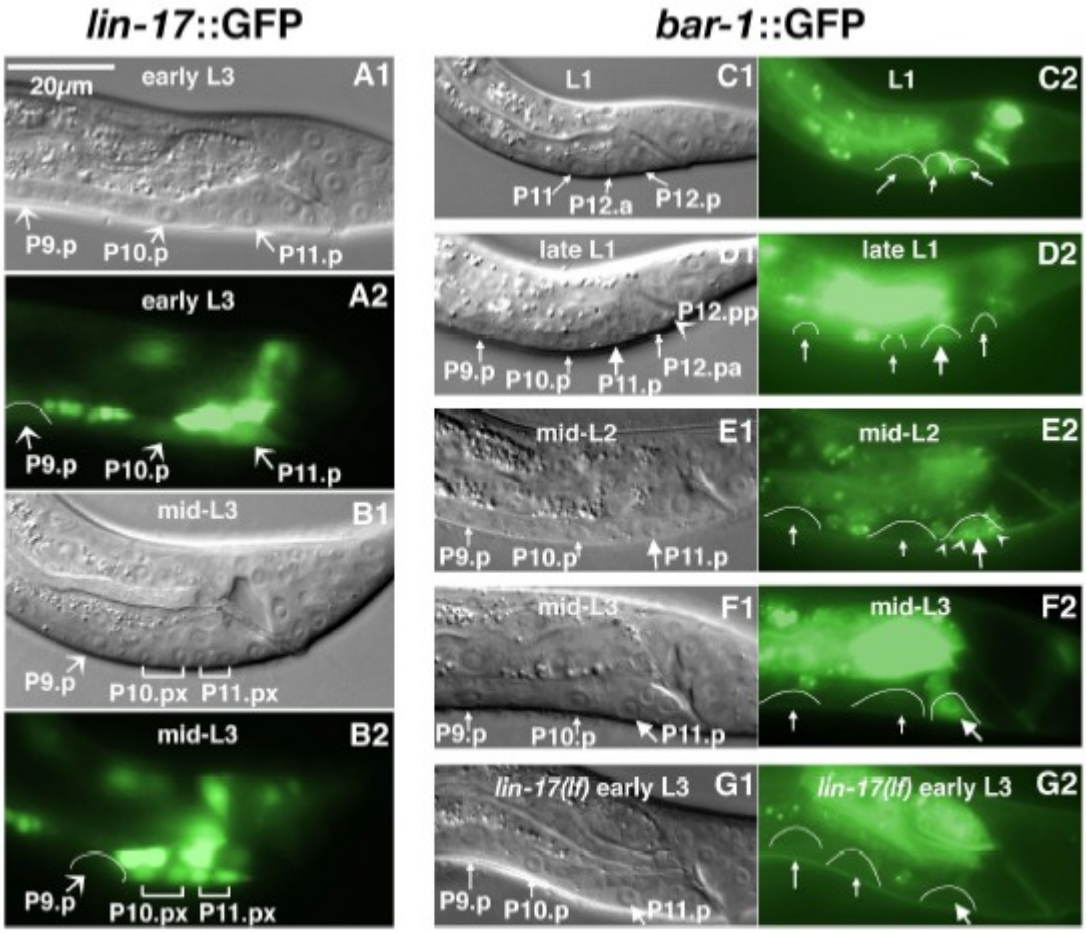


Fig. 6

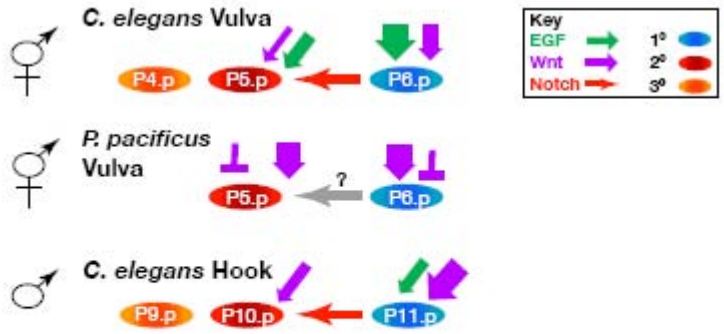


Fig. 7