EGF, WNT & HOX INTERACTIONS DURING PATTERNING OF *Caenorhabditis elegans* EQUIVALENCE GROUPS

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Adeline Seah

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ABSTRACT

During development, as a single-cell zygote divides multiple times to generate a complete organism, previously undifferentiated cells somehow acquire the correct fates. A group of cells that shares the same developmental potential is called an equivalence group. In *Caenorhabditis elegans*, the most well-characterized equivalence group is the hermaphroditic Vulval Precursor Cell (VPC) group. Epidermal Growth Factor (EGF) signaling specifies VPC fate partly by upregulation of *lin-39/SexcombsReduced/Hox5*, while Wnt signaling plays a minor role in vulval induction. EGF and Wnt signaling also act together to pattern the P11/12 equivalence group, present in both *C. elegans* hermaphrodites and males, by upregulating a different Hox gene, *egl-5/Antennapedia/UltrabithoraxHox6/8*, to specify P12 fate. Previous observations suggest that EGF or Wnt signaling may act through Hox genes to specify fate in two other *C. elegans* equivalence groups: the hook competence group (HCG) and γ/δ pair. I characterized the roles of EGF and Wnt signaling in the HCG and γ/δ pair, and found that upregulation of Hox genes is controlled by either pathway in each group.

I showed that the major hook inductive pathway involves the Wnt ligands and LIN-17/Fz, which specify the 1° and 2° HCG fates. Also, I identified a role for EGF signaling in specifying the 1° fate, although its role is only revealed when Wnt activity is compromised. I provided a link between *mab-5/Hox6/8* and Wnt signaling during normal hook development by determining that LIN-17 is required for *mab-5/Hox6/8* expression in P11.p.

In the γ/δ pair, I demonstrated that EGF signaling (through the LIN-31/Forkhead and LIN-1/ETS transcription factors) controls *ceh-13/Hox1* expression in γ. I did not find any evidence that Wnt signaling specifies the γ fate. Instead, I observed that *lin-44/Wnt, mom-2/Wnt* and *lin-17/Fz* are required to orient the γ mitotic spindle. In addition, TGF-β signaling (by *dbl-1/Dpp*) was previously reported to control γ expression of *ceh-13/Hox1*. I showed that *dbl-1* acts either downstream or in parallel to EGF signaling to specify the γ fate. I also found that *dbl-1/Dpp* does not appear to specify fates in the VPC and P11/12 equivalence groups, in which EGF signaling plays an important role, suggesting that TGF-β signaling contributes to the specificity of the γ fate.

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CHAPTER 1

Introduction

None of their grace beauty is suggested by a name that carries the stigma 'worm.'

–B.G. Chitwood[1]

To most people[2], worms are associated with nasty medical conditions, decomposing matter and to sum it up in a word, "gross." I have to confess, I used to belong to this majority[3]. After spending the last 6.5 years of my life studying *Caenorhabditis elegans*, a worm species belonging to the Nematoda phylum, however, I have developed an affection for *C. elegans* and much more cordial feelings toward other worms. In their defense, they are much more aesthetically pleasing than you would imagine. *C. elegans* is a microscopic species that grows to about 1 mm long in adulthood. Under the microscope, they look and move like tiny little snakes but lie on either their left or right side instead of on their dorsal side (or "belly down" position). Beyond appearances, *C. elegans* has been and continues to be an excellent organism in furthering our understanding of biology. In 2002, the Nobel Prize in Physiology or Medicine was jointly awarded to Sydney Brenner, H. Robert Horvitz, and John E. Sulston for establishing and using *C. elegans* as a model organism to study organogenesis and programmed cell death. Thanks to evolution, we can study genes and their functions in this noble worm and gain some possible insight into how these genes function in humans.

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[1] One of the early pioneers of nematology

[2] In certain parts of the world, worms are considered delicacies although many are actually insect larvae. Here are examples of "true" worms and the dishes they end up in: sandworm (a type of sipinculid) jelly is eaten in Xiamen, China, while the sex organs of the palolo worm (or *eunice viridis*) baked into a loaf with coconut milk and onions are enjoyed in Samoa.

[3] In college, I sat behind my lab partner while he dissected an earthworm, our assignment for the day. I like to think I've come a long way since then.

Before we go any further, there are several important terms and concepts to keep in mind:

"Mutations" are changes to the coding and non-coding regions of a gene that cause the gene product to act abnormally or not at all. "Alleles" refer to the different gene mutations that exist. "Null" or "loss-of-function (lf) alleles" completely remove any gene product from being produced. "Reduction-of-function (rf) alleles" reduce the activity or level of the gene product. "Gain-of-function (gf) alleles" increase the level or activity of the gene product activated or causes the gene product to acquire a novel function.

"Fate specification" refers to the process by which a cell integrates extracellular signals with intracellular factors to select a developmental outcome. In the situation where an external cue is responsible for fate specification, it is termed "induction." Prior to specification, cells of a particular equivalence group have to be competent to respond appropriately, meaning that they must have the ability to acquire distinct cell fates associated with that equivalence group.

During development, previously unspecified cells acquire the correct fates by the interaction of extrinsic signals with intrinsic factors (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). Groups of cells that have the same developmental potential are called equivalence groups (Cabrera et al., 1987; Kimble, 1981; Simpson and Carteret, 1990; Sulston and White, 1980). Within an organism, the same signaling pathways are often used multiple times during development to specify different fates. The invariant cell lineage of *C. elegans* provides us with a reproducible *in vivo* system of examining how signaling pathways interact at a single cell level.

Signaling pathways that instruct a cell to develop in a certain fashion often target "master control" genes such as Hox genes. Hox genes encode proteins, with a common 60 amino acid DNA-binding homeodomain, found in all metazoans[4] except sponges (Balavoine et al., 2002). In the late 1940s, Hox genes were first identified in *Drosophila melanogaster* mutant flies in which homeotic transformations had occurred, e.g., legs in place of antenna. Hox genes were found to pattern the anterior-posterior (AP) axis in most animals, and the genomic organization and expression pattern of Hox genes is conserved (Lemons and McGinnis, 2006; Lewis, 1978; Veraksa et al., 2000).

The Hox cluster in *C. elegans* is rudimentary and modified as compared to human and other vertebrate clusters, containing only six genes and an inversion between *ceh-13/labial/Hox1*[5] and *lin-39/Sexcombsreduced/Hox5* (Fig. 1) (Aboobaker and Blaxter, 2003). Although *C. elegans* Hox genes are highly divergent from *Drosophila* and vertebrate Hox genes, *Drosophila* Hox proteins can function in place of *C. elegans* Hox proteins to specify different cell fates (Hunter and Kenyon, 1995). Furthermore, Kuntz

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[4] AKA multicellular animals. Yes, that includes us humans.

[5] "Biologists would rather share their toothbrush than share a gene name" – Michael Ashburner, joint head of the European Bioinformatics Institute (Pearson, 2001).
Gene nomenclature can be confusing because each organism has it's own history and culture behind the naming of genes. One gene, *selectin L*, has 15 aliases. *Drosophila* geneticists have a penchant for more creative names that describe the mutant phenotype associated with a gene, such as *bazooka*, *comatose* and *mind-bomb* but do not give a clue about the gene product. Mouse scientists, on the other hand, have been more logical and guidelines for gene nomenclature state: A gene name should be specific and brief, conveying the character or function of the gene. Then, we have *C. elegans* nomenclature, which is mostly unhelpful, especially for the novice *elegans* grad student. *C. elegans* is similar to *Drosophila*, except the variety of mutant phenotypes in *C. elegans* is much smaller and the names aren't as fancy, explaining the 987 genes named *let(hal)-1* to *let-987* and 66 genes named *lin(eage defective)-1* to *lin-66*. Why is it important to know the names of the gene of interest in other organisms? Because as evolution would have it, knowing what your gene of interest does in other organisms helps you study the gene in your organism of interest. In my thesis, I will occasionally use the *C. elegans* name followed by the *Drosophila* name and finally the Human gene name (as I have here).

et. al (2008) identified regulatory elements in the *ceh-13-lin-39* intragenic region that were highly conserved between species and showed that the same elements from mouse drove the same expression pattern in *C. elegans* as the endogenous elements. This suggests that studying the regulation of Hox genes in C. *elegans* will likely shed light on Hox regulation in other species.

A. Extracellular Signals

In general, a signaling pathway consists of the signal (or ligand) that is presented to a cell. If the cell has the appropriate receptor for the ligand, upon ligand binding to the part of the receptor that lies outside the cell, the receptor activates downstream components within the cell to specify a certain response.

The Epidermal Growth Factor (EGF) Pathway

Binding of the EGF ligand to the EGF receptor causes the receptors to dimerize with each other. Subsequently autophosphorylation between the receptors occurs which leads to the recruitment of signaling components including the Adaptor proteins Growth Factor Receptor-Bound Protein-2 (GRB2) and Nck Adaptor Protein (Nck), Phospholipase-C-Gamma (PLC-Gamma), SHC (Src Homology-2 Domain Containing Transforming Protein), STATs (Signal Transducer and Activator of Transcription). EGF signaling often leads to changes in gene expression downstream of these diverse signaling pathways.

In particular, downstream of Grb2 and Son of Sevenless (SOS), Ras is a GTP-ase that activates a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK), Raf, at the

plasma membrane. Activated Raf phosphorylates a MAPKK, which in turn phosphorylates a MAPK. The final kinase in the cascade, MAPK, phosphorylates a range of downstream targets that can affect gene transcription and the activity of other proteins.

There is only one EGF ligand, LIN-3, and one EGF receptor, LET-23, in *C. elegans*. Activation of the Ras/MAPK cascade is required for several developmental events, e.g., vulva development and male tail development. The gene names of the other signaling components are as follows: *let-60/Ras*, *sem-5/Grb-2*, *lin-45/Raf*, *mek-2/MAPKK* and *mpk-1/MAPK*.

The Wnt Pathway

Wnts are a large family of secreted, hydrophobic, glycosylated ligands that are involved in diverse processes during development (Mikels and Nusse, 2006). Wnts can interact with a number of receptors including Frizzled (seven-pass transmembrane receptor), Ryk/Derailed (characterized by a Wnt Inhibitory Factor (WIF) domain), LRP5 and-6 (single pass transmembrane receptors of the low-density lipoprotein family) and ROR (receptor orphan tyrosine kinase). Downstream of the receptors, there are three Wnt subpathways: Wnt/β-catenin, Wnt/Ca^{2+} and Wnt/planar cell polarity (PCP) (Nelson and Nusse, 2004).

Here, we will limit the discussion to canonical Wnt/β-catenin signaling. In the absence of Wnt signaling, phosphorylation of β-catenin by casein kinase I (CKI) and glycogen synthase-3β (GSK-3β), which are bound to the Axin and adenomatous polyposis coli (APC) scaffolding proteins, causes ubiquitination and subsequent

degradation of β-catenin in the cytosol. Wnt stimulation leads to the inhibition of the Axin degradation complex, and β-catenin accumulates in the nucleus, allowing it to interact with the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor to regulate gene transcription.

In *C. elegans*, there are five Wnt genes (*lin-44*, *egl-20*, *mom-2*, *cwn-1*, *cwn-2*), four Frizzled Wnt receptors (*lin-17, mom-5*, *cfz-1* and *cfz-2),* one Ryk (*lin-18*) and one Ror (*cam-1*). *bar-1* is the *C. elegans* β-catenin that participates in canonical Wnt signaling.

B. Fate Specification in *Drosophila* Eye and Wing Imaginal Discs

Since *Drosophila melanogaster* was the first model organism established about a century ago, some of the most well-studied examples of fate specification of equivalence groups are found in this species. Examining the patterning of eye and wing imaginal discs in *Drosophila* have led to general principles of how cell fate is determined using a limited toolbox of signaling pathways during deveolopment.

I. The Eye Imaginal Disc

The *Drosophila* eye imaginal disc consists of a field of undifferentiated cells that initially possess the same developmental potential but subsequently acquire different cell fates due to spatially and temporally controlled cell-cell signaling events (Tomlinson and Ready, 1987). The eye disc gives rise to a highly ordered compound eye in the adult constituting about 800 ommatidia, each containing 8 light-sensing photoreceptor neurons (R1-8) and a complement of non-neural support cells arranged in a hexagonal shape.

During development, cellular differentiation is initiated by a morphogenetic furrow (MF) that moves posterior-to-anterior across the eye disc (Ready et al., 1976). Immediately posterior to the MF, 5-cell preclusters emerge in which the first photoreceptor neuron to be specified is R8, the founder cell of each ommatidium. Shortly after, R2 and R5 then R3 and R4 are determined within the precluster. At this point, all undifferentiated cells divide once followed by the specification of the cone cells and pigment cells.

All the eye cell fates except R8 require either EGFR signaling or both EGFR and Notch signaling (Voas and Rebay, 2004). It has been shown that in addition to instructional information provided by these pathways to specify the different eye fates, a combinatorial code of transcription factors (including Atonal, Rough, Prospero, D-Pax2, Lozenge, Spalt and Tramtrack88) affects the response of undifferentiated cells over time.

II. The Wing Imaginal Disc

The Drosophila wing imaginal disc is a single layered epithelium made up of about 50,000 cells (Crozatier et al., 2004). Very early on during development, the imaginal disc is divided into two groups of cells defined by their position — Anterior (A) or Posterior (P). Both groups of cells express different types of transcription factors (i.e. *engrailed*, *invected* and *cubitus interruptus*). Three secreted morphogens are used to pattern the wing imaginal disc: *wingless (wg)*, *decapentaplegic (dpp)* and *hedgehog (hh)*. The P cells secrete *hh*, which induces the A cells to express the growth factor *dpp*. *hh* and *dpp* control patterning of the A/P axis, while *wg* patterns the Dorsal/Ventral (D/V) axis. These three morphogens form concentration gradients and cells of the imaginal disc

are thought to detect their position within these gradients and generate the correct developmental fate accordingly (Gurdon and Bourillot, 2001).

C. Fate Specification in *C. elegans* Equivalence Groups

I. The P11/12 Equivalence Group

At hatch, the twelve P precursor cells form six bilateral pairs and each pair is named P1/2L, P1/2R, etc. (Sulston and Horvitz, 1977). During the mid-L1 stage, the P cells migrate into the ventral cord, line up along the anterior-posterior body axis and are subsequently renamed as Pn (n=1, 2, 3…, 12) cells (Fig. 2). The P11/12 pair exhibits a stereotypic migration where the left cell in the pair moves anteriorly to become P11, while the right cell moves posteriorly to become P12. If either cell of the P11/12 pair is killed prior to migration, the remaining cell always adopts the P12 fate (Sulston and White, 1980). Therefore, the P12 fate is the primary fate since it is the fate acquired by each cell in the equivalence pair if the other is absent. Two hours after migration into the ventral cord, both P11 and P12 divide once. In both hermaphrodites and males, the neuroblasts P11.a and P12.a subsequently generate several ventral cord neurons that are morphologically indistinguishable by Nomarski optics while P12.p divides once to generate P12.pa, which becomes the epidermal hyp12, and P12.pp, which undergoes cell death. P11.p fate, however, is sexually dimorphic: in hermaphrodites, P11.p does not divide and fuses to hyp7 in the late L1; in males, P11.p becomes part of the hook competence group (described in section III of this chapter).

EGF and Wnt signaling act synergistically to specify P12 fate (Jiang and Sternberg, 1998). Mutations in the EGF pathway components, *let-23/EGFR*, *sem-5/Grb2*

and *let-60/Ras*, that reduce EGF signaling activity cause P12-to-11 transformations. In contrast, excessive EGF signaling in *lin-15/lf* males results in P11-to-12 transformations. Although *lin-44/Wnt* and *lin-17/Fz* mutants exhibit similar defects to animals in which EGF activity is lowered, *lin-44/Wnt* overexpression does not have any effect on P12/11 fate. In addition, epistasis experiments indicate that Wnt signaling does not regulate *lin-3/EGF* activity to influence P12 fate. Therefore, the EGF and Wnt pathways appear to act in parallel to specify the P12 fate. EGF signaling is both necessary and sufficient to induce P12 fate, while Wnt signaling is necessary but not sufficient for P12 fate specification.

The Hox gene, *egl-5/AbdominalB/Hox9-13*, is upregulated by the EGF pathway (Jiang and Sternberg, 1998) and likely the Wnt pathway (Teng et al., 2004) during P12 fate specification (Fig. 3). *egl-5(null)* mutants exhibit P12-to-11 fate transformations (Chisholm, 1991; Kenyon, 1986). Furthermore, *egl-5* is sufficient to specify P12 fate in a reduced EGF signaling background, indicating that *egl-5* has an instructive as opposed to permissive role.

II. The hermaphrodite vulval precursor cells (VPCs)

The most well characterized equivalence group *in C. elegans* is the vulval precursor cells (VPCs) in hermaphrodites, which are also derived from the ventral P precursor cells (Sulston and White, 1980). After entering the ventral cord in the L1 stage, each Pn cell divides once to produce an anterior (Pn.a) and posterior (Pn.p) daughter. The VPCs, P(3-8).p, are located in the mid-body and each VPC has the potential to adopt either a 1°, 2° or 3° fate (Sulston and Horvitz, 1977). In about 50% of

hermaphrodites, however, P3.p fuses to the hyp7 epidermal syncytium without dividing, termed the “F” fate, prior to induction during the L2.

VPC Competence

VPCs must be maintained as individual epithelial cells to remain competent to respond to inductive signals. During the L1 stage, P(1-2).p and P(9-11).p are unable to bypass fusion in hermaphrodites because they do not express *lin-39/Sexcombsreduced/Hox5* (Salser et al., 1993). Expression of *lin-39/Scr*, however, prevents the VPCs from adopting the F fate. It is unknown what regulates *lin-39/Scr* expression at this stage. Later in development, *lin-39/Scr* activity is required again to prevent fusion to hyp7 in the VPCs. During the L2, Wnt signaling, through the downstream components *apr-1/APC* (Hoier et al., 2000) and *bar-1/β-catenin* (Eisenmann et al., 1998), and EGF signaling (Myers and Greenwald, 2007) act to establish VPC competence. Reduced Wnt signaling causes P(5-7).p to often adopt the 3° or F fate and P3.p, P4.p and P8.p to adopt the F fate, whereas the requirement for EGF signaling to maintain competence are only seen when Wnt activity is lower. Rf mutations of EGF pathway components enhance the F fate defects of Wnt signaling mutants. The Wnt pathway maintains *lin-39/Scr* expression to prevent fusion, while target genes of the EGF pathway are presently unknown (Eisenmann et al., 1998; Wagmaister et al., 2006). It is unknown which Wnt ligand(s) or receptor(s) act upstream of *bar-1* and *apr-1* in vulval competence.

VPC Induction

During the L3 stage, the major inductive signal, mediated by the EGF/Ras pathway, from the anchor cell (AC) causes the VPCs to divide during the L3 stage, generating a spatial pattern of 3°-3°-2°-1°-2°-3° (Kimble, 1981; Sommer, 2005; Sternberg, 2005; Sternberg and Horvitz, 1986). Rf mutations in *lin-3/EGF*, *let-23/EGFR*, *let-60/Ras*, *sem-5/Grb-2*, *mek-2/MEK* and *mpk-1/MAPK* as well as AC ablations cause a vulvaless (Vul) phenotype (Aroian et al., 1990; Beitel et al., 1990; Han and Sternberg, 1990; Hill and Sternberg, 1992; Kornfeld et al., 1995; Lackner et al., 1994; Sternberg and Horvitz, 1989; Wu and Han, 1994; Wu et al., 1995). Conversely, excessive EGF signaling results in a multivulva (Muv) phenotype. Mutations in the transcription factors *lin-1/ETS* and *lin-31/Forkhead*, which are phosphorylated by the EGF pathway, also cause vulval defects (Beitel et al., 1995; Miller et al., 1993; Tan et al., 1998).

In addition to EGF signaling, Wnt signaling has been shown to play a minor role during induction (Eisenmann et al., 1998). First, P(5-7).P in *bar-1/β-catenin* mutants occasionally adopt the 3° fate instead of 1° or 2° fates. Second, either overactivation of Wnt signaling in *pry-1/Axin* mutants or increased levels of a stabilized form of BAR-1/β-catenin causes an overinduction phenotype (Gleason et al., 2002).

Lateral signaling by the LIN-12/Notch pathway subsequently specifies the 2° fate and inhibits adjacent 1° fates (Ferguson et al., 1987; Greenwald et al., 1983; Sternberg and Horvitz, 1989). Consistent with Notch lateral signaling, the Delta/Serrate/Lag-2 (DSL) ligands, *lag-2*, *apx-1* and *dsl-1*, are upregulated by the EGF pathway in P6.p, which acquires the 1° fate (Chen and Greenwald, 2004).

lin-39/Scr also plays a role during vulval induction downstream of EGF signaling (Fig. 3) (Clandinin et al., 1997; Maloof and Kenyon, 1998). At the time of induction, EGF signaling upregulates *lin-39* expression, which is highest in P6.p (Wagmaister et al., 2006). Because Wnt signaling controls *lin-39* expression prior to induction, it is difficult to determine whether Wnt also is required for *lin-39* expression during the time of induction. Although loss of LIN-39 function can result in P(5-7).p adopting the 3° fate, increased levels of *lin-39* are not sufficient to induce vulval development when the AC (the source of the inductive signal) is removed. Therefore, EGF/Ras signaling probably has other targets besides *lin-39* which are required for vulval fate specification.

III. The male hook competence group (HCG)

The P descendants, P(9-11).p, in the male form the hook competence group (HCG) (Sulston and White, 1980). The hook is a reproductive structure that is required to locate the vulva and acts redundantly with the postcloacal sensillum. Sulston et al. (1980) demonstrated that P10.p gives rise to the major components of the hook sensillum, including a hook structural cell, two supporting cells (hook socket cell and sheath cell), and the hook sensory neurons HOA and HOB. Besides having similar developmental origins as the VPCs, the HCG also shares other similarities in terms of fate choices and use of LIN-12/Notch signaling (Greenwald et al., 1983). The adjacent anterior Pn.p (P10.p or P9.p) can substitute for the missing posterior cell if P11.p or P10.p is killed. 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°). Each HCG cell fate has a distinct cell division pattern and produces different types of descendants. In addition, the

Notch pathway is required for 2° fate specification and inhibits adjacent 1° fates (Greenwald et al., 1983).

Hook Competence

Another similarity between vulval and hook development is that a Hox gene is required to prevent fusion of the HCG to hyp7 during the late L1 in males (Kenyon, 1986; Salser et al., 1993). *mab-5/Antennapedia/Hox6-8* is expressed in the HCG during the L1 (Fig. 3), and P(9-11).p fuses to hyp7 in *mab-5(lf)* mutants. Unlike in the VPCs, in which fusion must be prevented a second time in the L2 so that cells remain competent to be induced, no factors appear to be required to prevent fusion of the HCG to hyp7 during the L2. There is also evidence that induction occurs during the mid-L2, suggesting that maintenance of the HCG as independent epithelial cells only occurs once during the L1. In males, MAB-5 is probably required for more than just preventing fusion during hook development (discussed further in the next section) because *lin-39/Scr* is expressed in P(3-6).p, allowing them to bypass fusion in the L1, but they do not adopt hook fates.

Hook Induction

The EGF pathway, which is the major inductive signal during vulval development, does not appear to be required for HCG specification (H. Chamberlin, personal communication). However, excessive EGF signaling in *lin-15(lf)* mutants results in P9.p adopting 2°-like fate instead of a 3° fate, indicating that EGF signaling can influence hook fates. Ablations of cells and different combinations of cells has failed to identify the source of the inductive signal during hook development (Herman, 1991;

Sulston et al., 1980). However, 1° and 2° HCG lineage defects in *lin-17/Fz(null)* males (Sternberg and Horvitz, 1988) suggests that Wnt signaling is involved in patterning the HCG (Fig. 3). Furthermore, increased canonical Wnt signaling in *pry-1/Axin(lf)* mutants causes anterior Pn.p cells to express HCG fates (H. Yu, personal communication). However, none of the Wnt ligands have been implicated in HCG specification.

Several observations suggest that *mab-5/Ant* acts a second time during hook development to specify hook fates. 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). Third, the ectopic hook phenotypes in *pry-1/lf* males is suppressed by a *mab-5(lf)* mutation (H. Yu, personal communication). Current evidence suggests that Wnt signaling upregulates *mab-5* in the HCG to specify hook fates. However, this has not been demonstrated.

Competence and induction have been discussed previously as separate events because they were characterized in the VPCs where defects in competence and induction are distinct (i.e. F fate versus 3° VPC fate). Since the 3° HCG fate is to either fuse to hyp7 or to divide once and fuse to hyp7, insufficient inductive signaling in the hook can result in a phenotype that is associated with competence defects in the vulva. This suggests that competence and induction may not necessarily be separate events.

IV. The B cell equivalence groups (α/β, γ/δ, ε/ζ)

The B cell is a male-specific blast cell that generates all the cells of the spicule. B.a generates four pairs of cells: the ventral pair (aa), the dorsal pair (pp) and two identical lateral pairs (ap/pa) (Chamberlin and Sternberg, 1993). Each pair of cells has an anterior (α, γ or ε) and a posterior fate (β, δ or ζ), and each fate produces different cell lineages and cell types (Fig. 2). The male-specific blast cells, U and F, are required to specify the anterior fate of each equivalence pair. In addition, both the U and F cells express the EGF ligand, LIN-3, and reduced activity of several genes in the EGF pathway (*lin-3/EGF*, *let-23/EGFR*, *sem-5/Grb2*, *let-60/Ras*, *lin-45/Raf*) causes abnormal anterior cell lineages (Chamberlin and Sternberg, 1994). Using lineage analysis to assay fate, a γ-to-δ fate transformation is observed in these EGF pathway mutants. The posterior daughter of the male-specific blast cell, Y, as well as LIN-12/Notch is required to specify the posterior fate of the γ/δ pair. In males in which Y.p is killed or in *lin-12(null)* males, a δ-to-γ fate transformation occurs. It is not known if Y.p is the source of the Notch ligand because Y.p is absent in *lin-12(null)* mutants. The fate transformations that occur in the absence of EGF or Notch signaling or in the cell ablation experiment described indicate that lateral signaling between the γ/δ pair is not most likely not necessary for fate specification.

Similar to the other equivalence groups where a Hox gene is expressed in the cell fate specified by EGF signaling, expression of the Hox gene, *ceh-13/labial/Hox1*, was observed in γ (Stoyanov et al., 2003). The TGF-β pathway components, *dbl-1/ dpp/TGF-β*, *sma-2/R-Smad*, *sma-3/R-Smad* and *sma-4/Co-Smad*, were reported to upregulate *ceh-*

13 in γ, implying a role for TGF-β signaling in specifying the γ fate (Fig. 3). *ceh-13* function during γ fate specification has not been examined.

V. Using Hox genes to generate specific outcomes downstream of the same signals

In the VPCs and the P11/12 equivalence group, EGF and Wnt pathways target different Hox genes to specify fate. As discussed above, there is evidence for the expression of a different Hox gene in each of the two equivalence groups, the HCG and the γ/δ pair, and the involvement of EGF and/or Wnt signaling to specify fate in the male B cell equivalence in these groups.

My overall aim was to characterize the roles of EGF and Wnt signaling in the HCG and γ/δ pair so as to understand better how signaling specificity is generated. I wanted to investigate signaling specificity at the pathway level and at the level of Hox regulation in the different *C. elegans* equivalence groups described above. In Chapter 2, I present my findings on EGF and Wnt signaling in the HCG. I provide evidence that Wnt signaling through the *lin-17/Fz* receptor specifies the 1° and 2° hook fates. Furthermore, I show that the role EGF signaling during 1° hook specification is only uncovered when Wnt signaling is reduced. Therefore, my data indicates that Wnt signaling is the major hook inductive signal and EGF signaling plays a minor role during hook development.

In Chapter 3, I examine EGF and Wnt signaling during γ fate specification. I demonstrate that the EGF pathway controls *ceh-13/labial* expression in γ. In addition, I show that certain Wnt signaling components are required to orient the γ mitotic spindle but do not appear to affect γ fate specification. Finally, I provide evidence that TGF-β

signaling does not appear to be required during vulval or P12 induction, suggesting that the TGF-β pathway may help provide specificity to the targets of EGF signaling during γ fate specification, such as *ceh-13*, as compared to the other targets required for vulval and P12 fate specification. Finally, I summarize my results in Chapter 4 and provide future directions for work in these areas I have worked on.

Figures

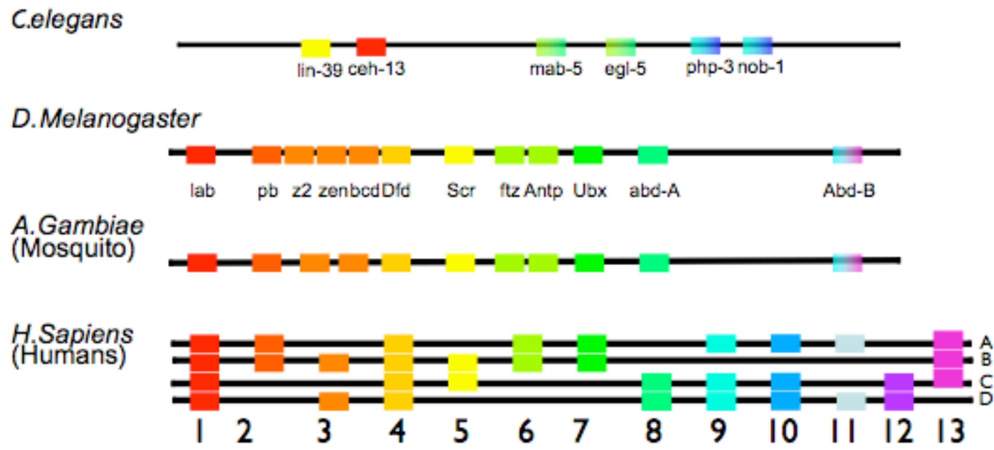

Fig. 1

Fig. 1. Conservation of genomic organization of Hox genes. It is difficult to define precise homology relationships for *mab-5*, *egl-5*, *nob-1* and *php-3*.

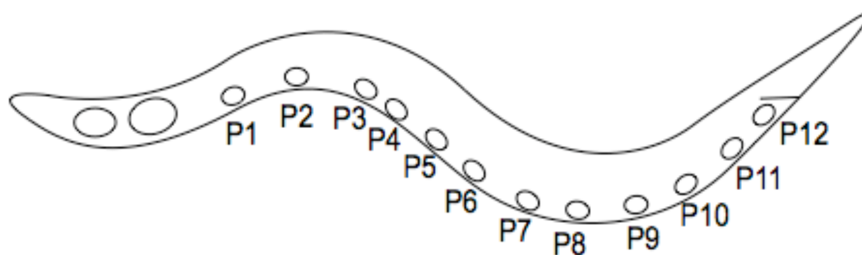

Fig. 2

Fig. 2. Arrangement of the twelve P cells in *C. elegans*.

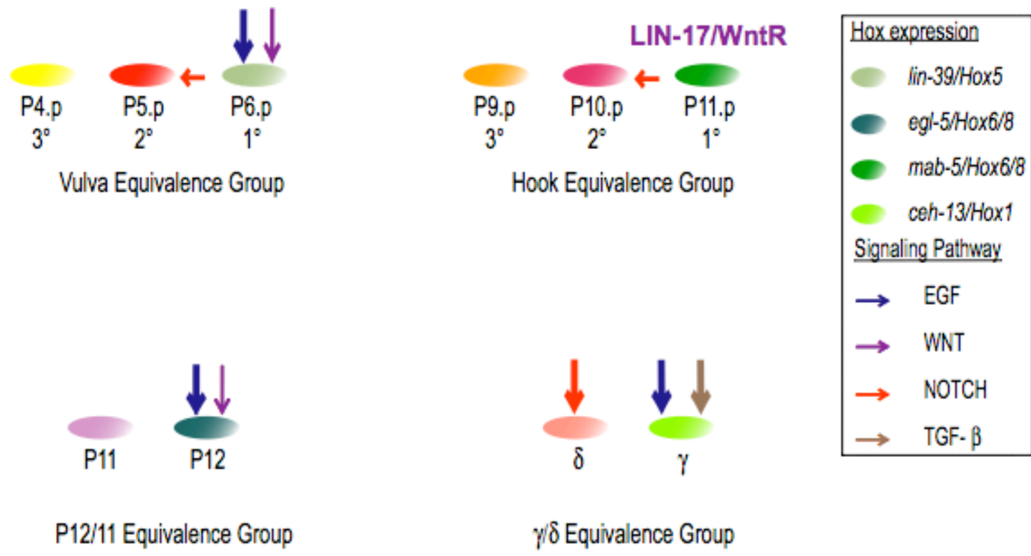

Fig. 3

Fig. 3. Comparison of the signaling pathways involved in the patterning of different equivalence groups. *lin-39/Hox5* and *egl-5/Hox6/8* are upregulated by EGF and Wnt in the vulval and P11/12 equivalence groups, respectively. *mab-5/Hox6/8* is expressed in the hook equivalence group, while *ceh-13/Hox1* is expressed in γ. EGF signaling has been shown to specify γ fate but regulation of *ceh-13/Hox1* expression has not been examined.

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CHAPTER 2

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

Adeline Seah[*], Hui Yu[*], Michael A. Herman, Edwin L. Ferguson, H. Robert Horvitz and Paul W. Sternberg

*Equal contribution

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Work presented in this paper began 25 years ago. Since then, the authors of this paper have contributed to the data presented in the paper as well as the writing of the paper. My contributions to the Hook paper are listed below:

1. Increased n for P10.p and P11.p single ablations in Table 1.
2. Scored hook lineages in *EGFR/let-23(sy97)* severe rf and *Grb-2/sem-5(n1619)* severe rf animals (Text)
3. Wnts are required to specify 1 °and 2 °fate (Table 4).
4. *lin-17/Fz* is required to specify 1 °and 2 °fate (Table 4).
5. True 1 º-to-3 °transformation in *lin-17/Fz* null mutants using *ajm-1*::GFP to assay fusion (Text & Fig. 5B).
6. Re-examined BAR-1—GFP expression (Fig. 6D & F).
7. Requirement for EGF signaling to specify 1 °fate only when Wnt signaling is compromised (Table 4).
8. Examined *mom-2/Wnt* and *lin-44/Wnt* expression in the male tail (Discussion).
9. Wnt signaling may be ancestral mode of patterning (Fig. 7)
10. Supplemental Tables 3-7.
11. Wrote most of the paper, responded to & dealt with reviewers comments.

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 ℃ 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

<u>Wnt signaling is required for 1 °and 2 °HCG fate specification and excecution</u>

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. During the L2, 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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Tables

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.

Figures

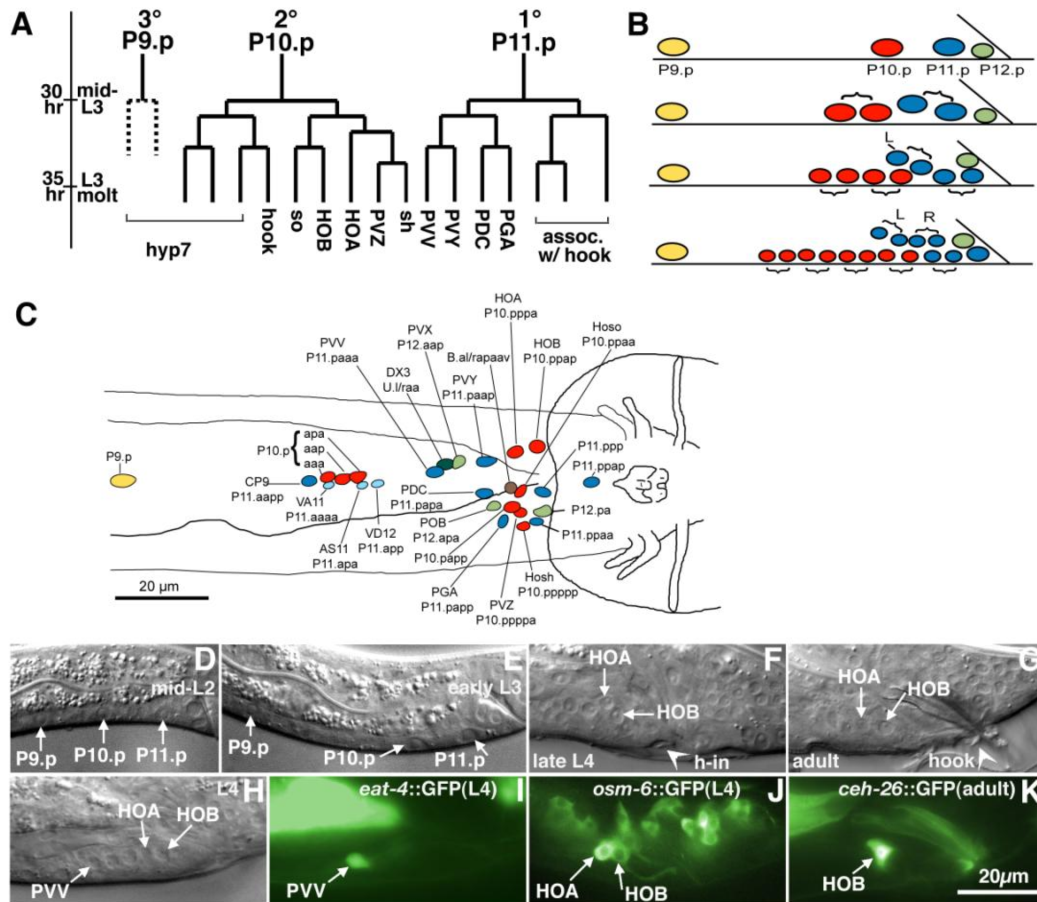


Fig. 1

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.

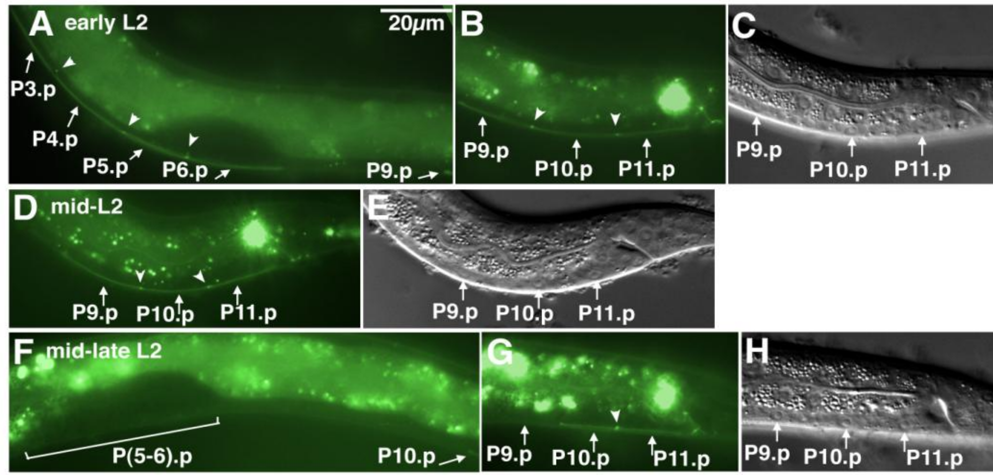


Fig. 2

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.

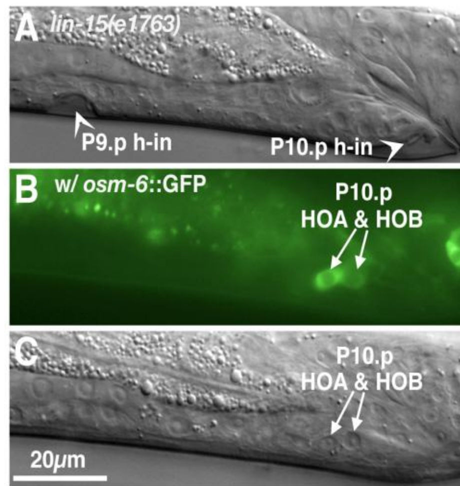


Fig. 3

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.

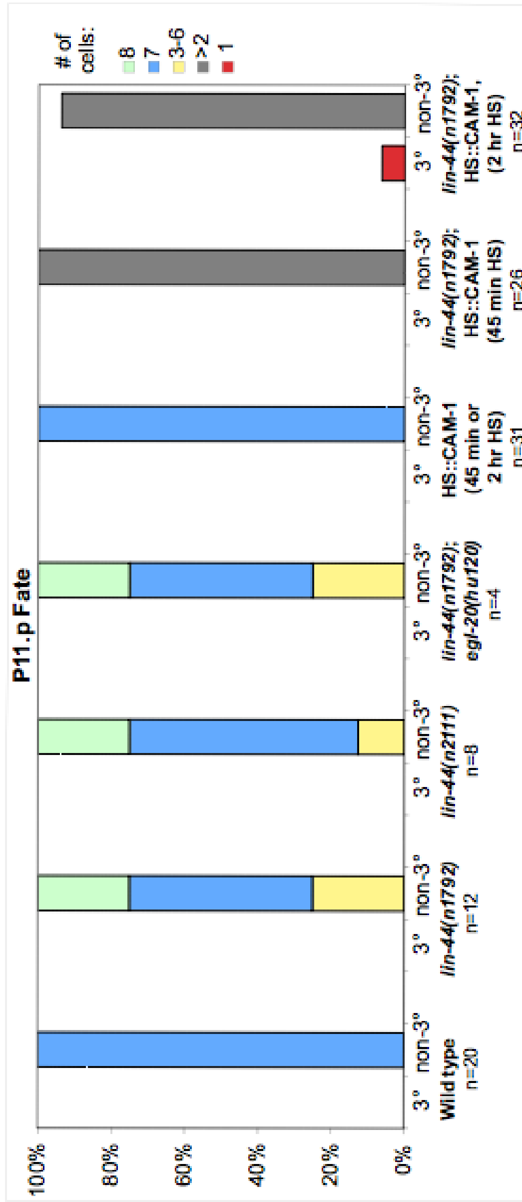
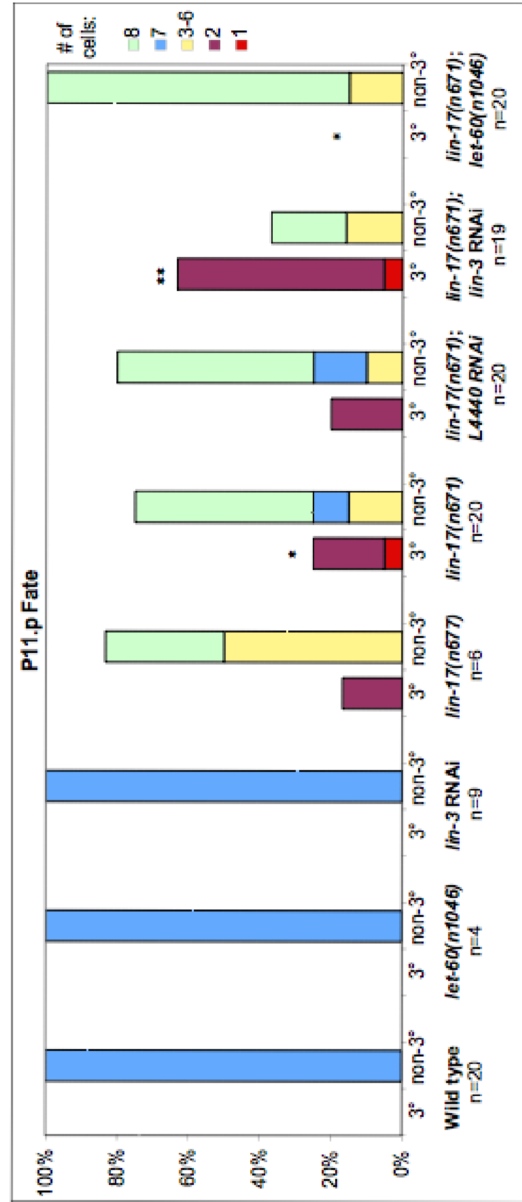


Fig. 4A



4B

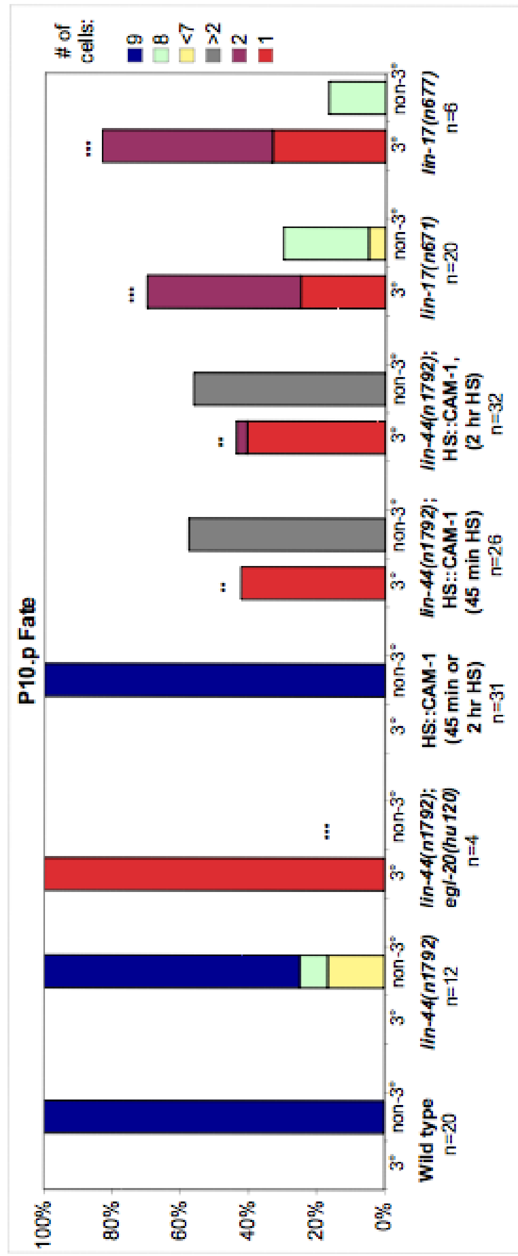


Fig. 4C

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(n677)* 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).

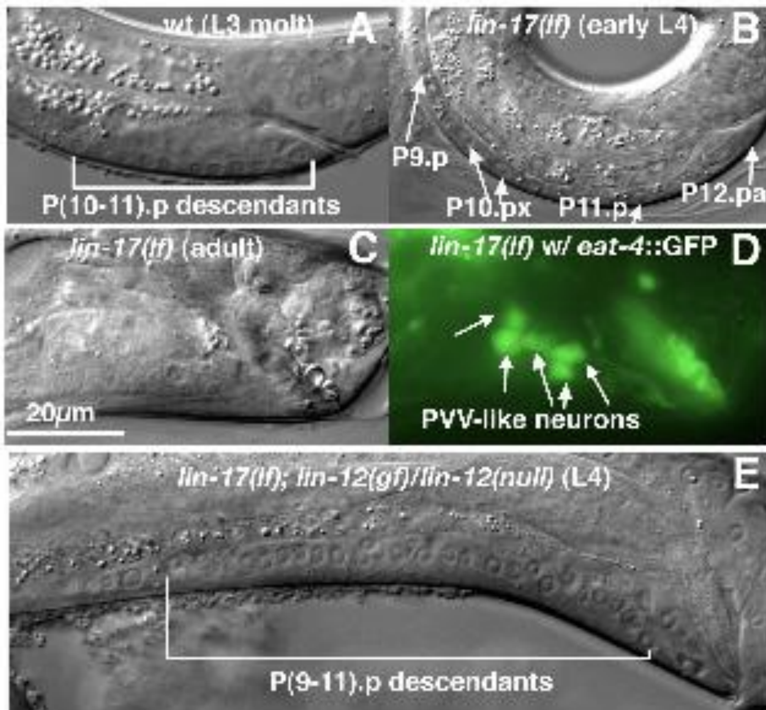


Fig. 5

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.

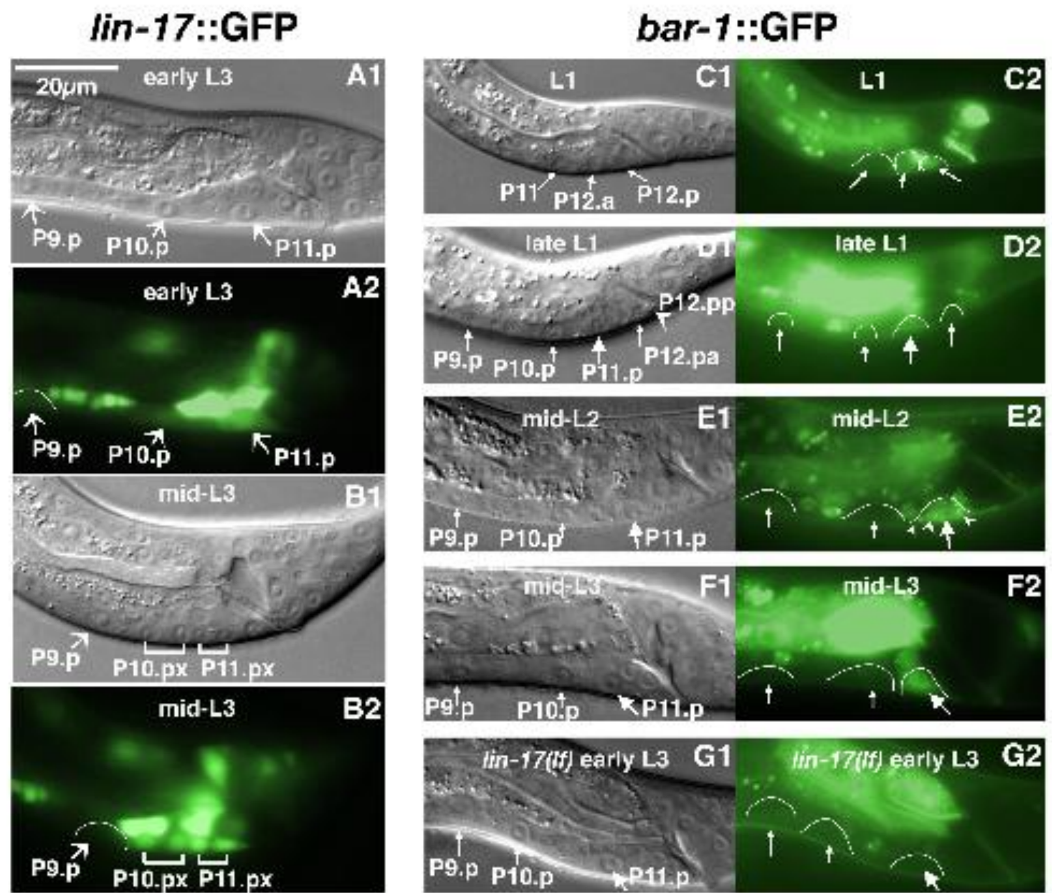


Fig. 6

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.

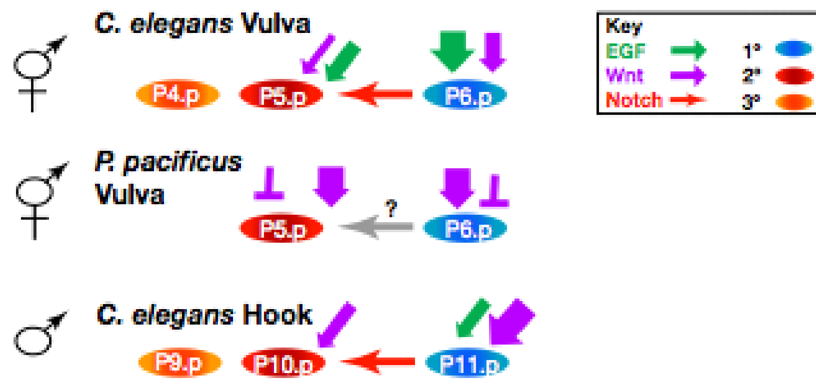


Fig. 7

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.

Supplemental Information

Table S1. HCG fate replacements after cell killing

Time of killing		**P11.p killed**[c]		**P10.p killed**[c]
Hours[a]	Stage[b]	P10.p → 1°	P9.p → 2°	P9.p → 2°
9-12	mid-L1	6/6	3/3	2/2
15	late-L1	1/1	0/1	ND
18-20	mid-L2	1/1	0/1	2/2
20-22	mid-L2	0/4	0/4	0/2
23-24	late-L2	ND	ND	0/2
25-26	early-L3	0/4	0/4	0/5

[a]: The time at which laser microsurgeries were performed as estimated by the cell lineages.
[b]: The larval stage (L1-L4) at which the laser microsurgical experiments were performed.
[c]: The number of times the indicated cell fate replacement was observed / the total number of animals subjected to laser microsurgeries.
ND: not done.

Table S2. Ectopic 2° HCG formation in *lin-15* mutants

Genotype[a]	2° P9.p (%)		2° P10.p (%)		Avg. hook-like structure	Avg. marker expression	n[b]
	Hook	Hook neuron (s)	Hook	Hook neuron(s)			
Wild type	0	NA	100	NA	1.0	NA	many
lin-15(e1763)[c]	73	NA	100	NA	1.7	NA	48
osm-6::GFP	0	0	100	100	1.0	1.0	many
osm-6::GFP; lin-15(e1763)	74	0	100	100	1.7	1.0	23
ceh-26::GFP	0	0	100	100	1.0	1.0	many
ceh-26::GFP; lin-15(e1763)	29	0	89	79	1.5	0.8	28[d]

[a] All strains contained *him-5(e1490)*. The integrated *osm-6*::GFP and *ceh-26*::GFP transgenes were *mnIs17* and *chIs1200*, respectively (Table S6).
[b] Number of animals scored.
[c] Only mutants with a wild-type P12 were scored.
[d] 3/28 *chIs1200; lin-15(e1763)* males had neither the hook structure nor the HOB hook neuron; and another 3/28 *lin-15* mutants lacked the hook neuron whether or not an ectopic hook was present, indicating a low frequency of deficient 2° fate induction in this strain.

Table S3. P(9-11).p cell lineages in Wnt mutants

Background[a]	No. of animals	No. of descendants[b]		
		P10.p	P11.p	P12.p
Wild type	**9**	9	7	-
egl-20(hu120)	**29**	9	7	-
cwn-2(ok895)	**20**	9	7	-
lin-44(n1792)	**1**	5	5	-
	1	6	5	-
	1	8	5	-
	4	9	7	-
	3	9	8	-
	2	9	7[c]	-
lin-44(n2111)	**1**	8	6	-
	5	9	7	-
	1	5	8	-
	1	9	8	-
cwn-1(ok546); *cwn-2(ok895)*	**10**	9	7	-
cwn-1(ok546); *egl-20(n585)*	**11**	9	7	-
lin-44(n1792); egl-20(hu120)	**1**	1	4	-
	1	1	7[d]	-
	1	1	7[e]	-
	1	1	8	-
	2	1	8	8

[a] All strains contained *him-5(e1490)* in the background. Only mutants with a wild-type P12 were scored.

[b] Nematodes were mounted in M9 buffer on a 3% agar slab. Lineages were observed for animals until the end of the L3 lethargus. Air temperature was maintained between 19 and 22°C.

[c] Although P11.p generated a wild-type number of cells, P11.pp polarity was reversed such that P11.ppp divided in the same manner as wild-type P11.ppa and P11.ppa did not divide similar to wild-type P11.ppp.

[d] Although P11.p generated a wild-type number of cells, P11.p polarity was reversed such that P11.pp divided in the same manner as wild-type P11.pa and P11.pa divided in the same manner as wild-type P11.pp.

[e] Although P11.p generated a wild-type number of cells, the division plane of P11.pa was abnormal.

Table S4. P(9-11).p cell lineages in *lin-17* mutants

Genotype[a]	No. of animals	No. of cells generated[b]		
		P9.p[c]	P10.p	P11.p
Wild type	**9**	1	9	7
lin-17(n671)[d]	**1**	1	2	1
	1	1	1	2
	3	1	2	2
	1	1	1	4
	1	2	1	5
	1	1	1	6
	1	1	2	7
	1	1	1	8
	2	1	2	8
	2	2	2	8
	1	1	5	8
	1	2	8	7
	1	1	8	8
	3	2	8	8
lin-17(n677)[d]	**1**	2	2	2
	1	1	1	6
	1	1	2	6
	1	1	1	8
	1	2	2	8
	1	1	8	6

[a] All strains contained *him-5(e1490)* in the background. *n671* and *n677* are null alleles of *lin-17*(Ferguson and Horvitz, 1985; Sawa et al., 1996).

[b] Nematodes were mounted in M9 buffer on a 3% agar slab. Lineages were observed for animals until the end of the L3 lethargus. Air temperature was maintained between 19 and 22°C.

[c] The single round of P9.p division was observed more often in *lin-17(lf)* mutants than in wild-type males (0/9 in wild-type and 7/20 in *lin-17(n671)* mutants). This difference might reflect lack of lateral signaling from a normal 2 °P10.p lineage.

[d] Because LIN-17 is partially required for P12 fate specification and P12 defects can lead indirectly to HCG defects, we studied only HCG lineages in mutants that had a wild-type P12.

Table S5. Interaction of EGF and Wnt signaling during HCG induction

Background[a]	**No. of animals**	**No. of descendants**[b]	
		P10.p	**P11.p**
Wild type	**9**	9	7
lin-3 RNAi[c]	**9**	9	7
lin-17(n671); L4440 vector RNAi[c]	**1**	1	2
	3	2	2
	1	2	5
	1	2	6
	1	1	7
	1	2	7
	7	2	8
	1	6	7
	1	5	8
	3	8	8
lin-17(n671); lin-3 RNAi[c]	**1**	1	1
	5	1	2
	6	2	2
	1	1	4
	1	2	5
	1	2	6
	4	8	8
lin-17(n671); let-60(n1046gf)[c]	**1**	1	5
	1	4	8
	1	7	6
	1	8	6
	16	8	8
let-60(n1046gf)[c]	**4**	9	7

[a] All strains contain *him-5(e1490)*.
[b] Nematodes were mounted in M9 buffer on a 3% agar slab. Lineages were observed for animals until the end of the L3 lethargus. Air temperature was maintained between 19 and 22°C.
[c] Because LIN-17 is partially required for P12 fate specification and P12 defects can lead indirectly to HCG defects, we studied only HCG lineages in mutants that had a wild-type P12.

Table S6. Alleles and Transgenes.

Allele/Transgene	LG	notable feature	reference
cwn-1(ok546)	I	loss-of-function	Zinovyeva and Forrester, 2005
lin-17(n671)	I	loss-of-function	Ferguson and Horvitz, 1985
lin-17(n677)	I	loss-of-function	Ferguson and Horvitz, 1985
lin-44(n1792)	I	loss-of-function	Herman et al., 1995
lin-44(n2111)	I	loss-of-function	Herman et al., 1995
let-23(n1045)	II	temperature-sentitive	(Aroian et al., 1990; Ferguson and Horvitz, 1985)
let-23(sy97)	II	reduction-of-function	Aroian et al., 1990
let-23(sa62)	II	gain-of-function	Katz et al., 1996
unc-32(e189)	III	marker	Brenner, 1974
dpy-19(e1259)	III	marker	Brenner, 1974
lin-12(n137)	III	gain-of-function	Greenwald et al., 1983
lin-12(n137n720)	III	null	Greenwald et al., 1983
lin-12(n676n909)	III	null	Greenwald et al., 1983
him-8(e1487)	IV	Him	Hodgkin et al., 1979
egl-20(n585)	IV	reduction-of-function	Maloof et al., 1999
egl-20(hu120)	IV	loss-of-function	Coudreuse et al., 2006
cwn-2(ok895)	IV	loss-of-function	Zinovyeva and Forrester, 2005
let-60(n1046)	IV	gain-of-function	Ferguson and Horvitz, 1985
mom-2(or42)	V	reduction-of-function	Thorpe et al., 1997
him-5(e1467)	V	Him	Hodgkin et al., 1979
him-5(e1490)	V	Him	Hodgkin et al., 1979
bar-1(ga80)	X	null	Eisenmann et al., 1998
dpy-6(e14)	X	marker	Brenner, 1974
unc-9(e101)	X	marker	Brenner, 1974
lin-15(n765)	X	temperature-sentitive	Ferguson and Horvitz, 1985
lin-15(n309)	X	null	Ferguson and Horvitz, 1985
lin-15(e1763)	X	null	Ferguson and Horvitz, 1985
syIs78	I	AJM-1–GFP	Gupta et al., 2003
chIs1200	III	*ceh-26*::GFP	Yu et al., 2003
mnIs17	V	*osm-6*::GFP	Collet et al., 1998
adIs1240	X	*eat-4*::GFP	Lee et al., 1999
gaIs45	X	BAR-1–GFP	Eisenmann et al., 1998
syEx556		*cwn-1*::GFP	T. Inoue, unpublished
syEx566		*cwn-2*::GFP	T. Inoue, unpublished
syEx631		*cwn-2*::GFP	T. Inoue, unpublished
syEx664		*mom-2*::GFP	Inoue et al., 2004
syEx670		*lin-44*::GFP	Inoue et al., 2004

syEx676	*lin-17*::GFP	B. P. Gupta and P.W. Sternberg, unpublished
syEx710	HS::CAM-1	Green et al., 2008

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Table S7. List of Strains

Strain name	Genotype
EU384	*+/ nT1[let-?(m435)]*; *dpy-11(e224) mom-2(or42)/ nT1*[a]
VC636	*cwn-2(ok895)*[a]
CB1490	*him-5(e1490)*
CB1489	*him-8(e1489)*
PS3380	*mnIs17 him-5(e1490)*
PS3842	*bar-1(ga80)*; *him-5(e1490)*
EM665	*chIs1200*; *him-5(e1490)*
PS1318	*dpy-10(e128)*; *let-60(n1046)*[a]
PS3420	*dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909)*; *mnIs17 him-5(e1490)*
PS4145	*him-5(e1490)*; *adIs1240*
PS4316	*him-5(e1490)*; *gaIs45*
PS1309	*him-5(e1490)*; *lin-15(e1763)*
PS4579	*let-23(sa62) unc-4(e120)/mnC1*; *him-5(e1490)*
PS1477	*let-23(sa62) unc-4(e120)/mnC1*; *lin-15(e1763)*[a]
MT309	*lin-15(n309)*; *him-5(e1490)*
PS4277	*lin-17(n671)*; *him-5(e1490)*
PS18	*lin-17(n677)*; *him-5(e1490)*
PS4777	*lin-17(n671)*; *mnIs17 him-5(e1490)*
PS5609	*lin-44(n1792)*; *him-5(e1490)*
PS5256	*lin-44(n2111)*; *syIs145*; *him-5(e1490)*
PS4657	*syIs78*; *him-5(e1490)*
PS4290	*unc-119(ed4)*; *him-5(e1490)*; *syEx566*
PS4838	*unc-119(ed4)*; *him-5(e1490)*; *syEx664*
PS4840	*unc-119(ed4)*; *syEx676*[a]
PS4287	*chIs1200*; *him-5(e1490)*; *bar-1(ga80)*
PS3513	*chIs1200*; *him-5(e1490)*; *lin-15(e1763)*
PS5554	*cwn-1(ok546)*; *cwn-2(ok895)*; *him-5(e1490)*
PS5207	*cwn-1(ok546) syIs145*; *egl-20(n585)*; *him-5(e1490)*
PS3512	*let-23(sa62)*; *let-60(n1046)*; *mnIs17 him-5(e1490)*
PS4738	*lin-17(n671)*; *him-5(e1490); adIs1240*
PS4841	*lin-17(n671) syIs78*; *him-5(e1490)*
PS4288	*lin-17(n671)*; *chIs1200; him-5(e1490)*
PS4418	*lin-17(n671)*; *him-5(e1490)*; *gaIs45*
PS5608	*lin-44(n1792)*; *egl-20(hu120)*; *him-5(e1490)*
PS5767	*lin-44(n1792)*; *him-5(e1490)*; *syEx710*
PS3388	*mnIs17 him-5(e1490)*; *lin-15(e1763)*
MT2351	*unc-32(e189) lin-12(n137 n720)*; *lin-15(n309)*; *him-5(e1467)*
PS4720	*dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909)*; *mnIs17 him-5(e1490)*; *adIs1240*
PS5275	*lin-17(n671)*; *let-60(n1046)*; *him-5(e1490)*
PS4623	*lin-17(n671)*; *dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909)*; *mnIs17 him-5(e1490)*
PS4753	*lin-17(n671)*; *dpy-19(e1259) lin-12(n137)/unc-32(e189) lin-12(n676n909)*; *mnIs17 him-5(e1490)*; *adIs1240*

[a] Strains used to construct lines which contained either *him-5(e1490)* or *him-8(e1489)*

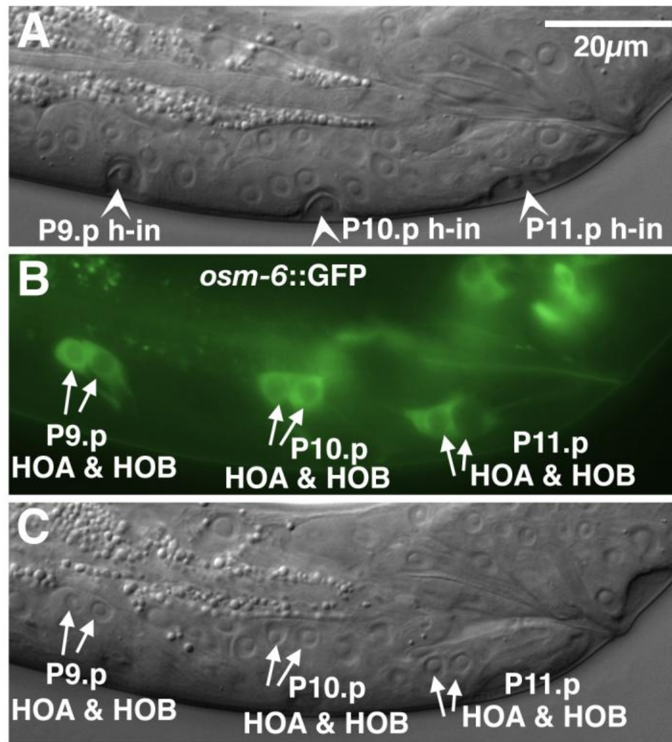
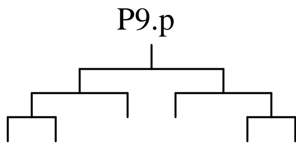


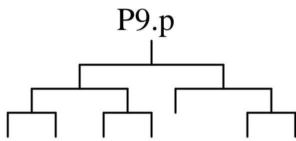
Fig. S1. Specification of the 2° HCG fate by LIN-12 lateral signaling. (A-C) Ectopic expression of the 2° fate by P(9-11).p in response to activated LIN-12 signaling. An L4 *lin-12(gf)/lin-12(null)* male with three hook invaginations (arrowheads) (A, Nomarski) and three pairs of HOA and HOB hook neurons as visualized by the *osm-6*::GFP marker (arrows) (B, fluorescence; C, Nomarski). hIn, hook invagination. Scale bar, 20 μm. Left lateral views.

Fig. S1

2°-like lineage

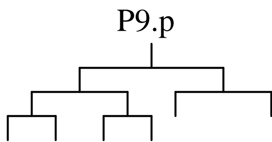


Made a hook-like structure

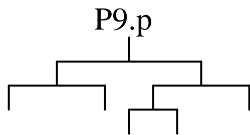


Made a hook structure

1°-like lineage



Did not form a hook



Did not form a hook

Fig. S2. P9.p lineages of *lin-15(n309)* males

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CHAPTER 3

EGF and Wnt signaling during patterning of the *C. elegans* Bγ/δ Equivalence Group

Adeline Seah and Paul W. Sternberg

Abstract

During development, different signaling pathways interact to specify fate by regulating transcription factors necessary for the correct development response. In *C. elegans*, the EGF-Ras and Wnt signaling pathways have been shown to interact to specify cell fate in three equivalence groups: the vulval precursor cells (VPCs), the hook competence group (HCG) and P11/12. In the VPCs, HCG and P11/12 pair, EGF and Wnt signaling upregulate different Hox genes, which also function during fate specification. In the male, EGF-Ras signaling is required to specify the γ fate of the γ/δ equivalence pair, while Notch signaling is required for δ fate specification. Previous work showed that TGF-β signaling by *dbl-1/dpp* controls *ceh-13/labial/Hox1* expression in γ. Here, we show that EGF-Ras signaling is also required for γ expression of *ceh-13/labial/Hox1*. We also find that *lin-1/ETS* and *lin-31/Forkhead* function downstream of the EGF pathway to control *ceh-13* expression and therefore γ fate specification. We have also identified a role for Wnt signaling: *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* act to orient the γ mitotic spindle. Finally, our results suggest that *dbl-1/dpp* is not required for VPC and P12 specification.

Introduction

During development, fate specification within equivalence groups (a set of cells with similar potential) often requires extracellular cues provided by surrounding cells (Blair and Weisblat, 1984; Campos-Ortega and Knust, 1990; Eisen, 1992; Kimble, 1981; Weisblat and Blair, 1984). The response elicited by a particular signaling pathway is context-specific: the fate acquired by a cell depends on its developmental history (i.e., the genes expressed by a cell) as well as the presence of other external signals. One mechanism by which signaling pathways specify fate is by regulating master control genes that initiate expression of a battery of genes required for a particular fate. Hox genes are a class of master regulators that pattern the anterior-posterior axis of metazoans during embryogenesis. In *C. elegans*, there is accumulating evidence that different Hox genes are upregulated by Wnt and EGF-Ras signaling in different equivalence groups.

EGF and Wnt signaling act together to specify fates within three different equivalence groups in *C. elegans*: the vulval precursor cells (VPCs), the hook competence group (HCG) and the P11/12 group (Eisenmann et al., 1998; Jiang and Sternberg, 1998; Sternberg, 2005; Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977). Each of these equivalence groups involves the patterning of Pn cells. During the first larval (L1) stage, each postembryonic Pn (n=1, 2, 3, …, 12) precursor cell is positioned along the anterior-posterior axis on the ventral epithelium and divides to produce an anterior (Pn.a) and a posterior daughter (Pn.p). The P11/12 equivalence group is found in both hermaphrodites and males, and EGF and Wnt signaling are required to specify the P12 fate, which is the 1° fate. In hermaphrodites, the central Pn.p cells, P3-8.p, comprise the VPCs, which can each adopt a 1°, 2° or 3° vulval fate. The

EGF-Ras pathway induces the 1° VPC fate while Wnt signaling plays a minor role in induction. In males, the posterior Pn.p cells, P9-11.p, form the HCG that gives rise to the hook (a male reproductive structure involved in vulva location behavior). Similar to the VPCs, there are 3 HCG fates: 1°, 2° or 3°. However, in contrast to vulval development, Wnt signaling is the major inductive signal during hook development, specifying the 1° and 2° HCG fates (Yu et al., 2008). A role for EGF-Ras signaling in HCG specification is only observed when Wnt signaling is compromised. In addition, LIN-12/Notch signaling specifies both the 2° VPC and 2° HCG fates by lateral signaling (Greenwald et al., 1983; Sternberg and Horvitz, 1989).

Different Hox genes are required to specify vulval and P12 fates downstream of the EGF and Wnt pathways. Specifically, *lin-39/SexcombsReduced/Hox5* is upregulated in the VPCs by EGF and Wnt signaling, while *egl-5/Antennapedia/Ultrabithorax/Hox6/8* is expressed in P12 and upregulated by EGF, and most likely Wnt signaling, in P12.pa (a descendant of P12) (Eisenmann et al., 1998; Jiang and Sternberg, 1998; Wagmaister et al., 2006). Overexpression of *lin-39* or *egl-5* is also partially sufficient to specify vulval or P12 fates, respectively. Although a role for MAB-5/Antennapedia/Ultrabithorax/Hox6/8 has not been shown in the HCG, *mab-5* is expressed in the HCG and is regulated by Wnt signaling (Appendix). Furthermore, increased Notch signaling in *lin-12(gf)* males results in P(3-8).p acquiring vulval fates and P(9-11).p adopting hook fates, implying that P(3-8).p and P(9-11).p have different propensities to generate vulval and hook lineages, respectively (Greenwald et al., 1983). Overexpression of MAB-5 in *lin-39(rf)* hermaphrodites also causes P(5-7).p to display hook-like features (Maloof and Kenyon, 1998). Taken together, these observations

suggest, that similar to vulval and P12 development, a Hox gene (*mab-5*) may be required to specify HCG fates. A fourth Hox gene, *ceh-13/labial/Hox1*, is expressed in another equivalence group that requires EGF signaling for fate specification: the γ/δ pair generated by the B cell, a male-specific blast cell.

The B cell gives rise to the male copulatory spicules (Sulston et al., 1980; Sulston and Horvitz, 1977). B.a generates 10 cells, of which 4 pairs form the γ/δ, α/β and the two ε/ζ equivalence groups (Fig. 1A). Each cell type has a distinct division pattern. In particular, γ divides in a longitudinal fashion and produces six progeny where one dies, while δ divides in a transverse fashion once to produce two progeny. Of the five remaining γ progeny, two are neuronal support cells and three are proctodeal cells; both δ progeny are proctodeal cells. Several findings indicate that EGF signaling specifies the anterior cell fate of each equivalence pair. Ablation of the male-specific blast cells, U and F, which are one source of anterior *lin-3/EGF*, can cause the anterior cell to adopt the posterior fate (Chamberlin and Sternberg, 1993; Chamberlin and Sternberg, 1994; Hwang and Sternberg, 2004). In addition, reduction-of-function (rf) mutations in *lin-3/EGF*, *let-23/EGFR*, *sem-5/Grb2*, *let-60/Ras* and *lin-45/Raf* cause anterior-to-posterior fate transformations within each equivalence group (Chamberlin and Sternberg, 1994). Conversely, excessive EGF signaling due to ectopic expression of the EGF domain using a heat-shock transgene or a *lin-15(null)* mutation causes the posterior cell to acquire the anterior fate. Fate transformations in these experiments were assayed based on the number of progeny generated by each fate and the orientation of the first division after induction for the γ/δ pair (Fig. 1B).

The γ/δ pair was characterized in further detail by the ablation of the posterior daughter of Y, another male-specific blast cell, that indicated a role for Y.p in promoting the posterior fate, δ. In addition, when U and F are absent or when U, F and Y.p are absent, increased LIN-12/Notch signaling in *lin-12(gf)* males causes γ-to-δ fate transformations. These results suggest that LIN-12/Notch is sufficient to specify the δ fate in the absence of Y.p. Conversely, reduced LIN-12/Notch signaling in *lin-12(null)* males resulted in δ-to-γ fate transformations. However, since Y.p is absent in *lin-12(null)* males, it is not possible to establish whether Y.p is sufficient to specify the δ fate in these mutants. In the absence of U, F and Y.p, the γ/δ equivalence pair is still able to express the γ and δ fates, suggesting that other external cues act to specify these fates. Furthermore, reduced EGF signaling did not cause a γ-to-δ fate transformation in all animals: partial fate transformations were observed in which the presumptive γ cell either divided in a wild-type longitudinal fashion but produced four progeny (less than the wild-type number of six progeny) or divided in a transverse fashion (δ-like) but produced more than two progeny (γ-like). Unfortunately, it is not possible to determine γ fate specification in mutants carrying null alleles of EGF signaling pathway components because EGF signaling is required for viability at an earlier larval stage.

Stoyanov et al. (2003) reported that *ceh-13/labial* was expressed in γ and that expression required *dbl-1/ dpp/TGF-β*, *sma-2/R-Smad*, *sma-3/R-Smad* and *sma-4/Co-Smad* — components of the TGF-β pathway that also regulates the Sma/Mab pathway in *C. elegans* (Morita et al., 1999; Savage et al., 1996; Suzuki et al., 1999). Moreover, in *Drosophila*, the TGF-β, EGF and Wnt pathways regulate *labial* expression during midgut morphogenesis (Immergluck et al., 1990; Panganiban et al., 1990; Szuts et al., 1998).

Therefore, we wished to investigate whether EGF and Wnt signaling also regulate *ceh-13/labial* expression. And conversely, since the TGF-β pathway was reported to regulate *ceh-13/labial* expression, we also examined whether TGF-β signaling is involved in VPC, HCG and P12 specification.

Here, we show that the EGF pathway is required for the expression of *ceh-13/labial/Hox1* in γ. In addition, *lin-1/ETS* and *lin-31/Forkhead*, transcription factors which act downstream of EGF signaling during vulval development, are required for γ fate specification. We also provide evidence that *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* control the spindle orientation of γ during division but are not required for *ceh-13* expression. Using a Wnt activated transcriptional reporter, POPTOP, we found that *lin-44/Wnt* and *lin-17/Fz* probably orient the γ mitotic spindle without requiring a specific transcriptional output. Our results indicate that EGF and TGF-β signaling by the *C. elegans dpp/BMP* ortholog, *dbl-1*, specify the γ fate and that TGF-β signaling likely acts downstream or in parallel to the EGF pathway. By contrast, we show that *dbl-1/TGF-β* signaling appears to have no role in VPC and P12 specification. Since the other equivalence groups also use the EGF and Wnt pathways, TGF-β signaling may account for the specificity of the γ fate.

Materials and Methods

Genetic methods and strains

Strains were grown at 20°C as described in Brenner (1974), unless otherwise indicated. All strains used contain the *him-5(e1490)* mutation (Hodgkin et al., 1979) which has been omitted from the following description of the strains used:

PS21: *let-23(sy1)*, PS4807: *syIs145* [Int *ceh-13*::GFP] (described below), PS4814: *syIs145*; *let-60(n1046gf)*, PS5000: *syIs145*; *lin-15(e1763)*, PS5014: Ex[HS::*lin-3*; *pha-1*(+); *myo-2*::GFP], PS5026: *syIs145*; *lin-1(e1777)*, PS5031: *syIs145*; *sem-5(n1619)*, PS5032: *syIs145*; *let-60(n2021)*, PS5087: *syIs145 lin-31(bx31)*, PS5101: *syIs145 lin-31(n301)*, PS5193: *lin-17(n698)*; *syIs145*, PS5207: *syIs145 cwn-1(ok456); egl-20(n585)*, PS5208: *syIs145*; *lin-1(n1790gf)*, PS5256: *lin-44(n2111)*; *syIs145*, PS5501: *syIs145*; *dbl-1(wk70)*, PS5333: *unc-119(ed4*); *syIs188* [CherryPOPTOP (described below), *unc-119(+)*], PS5552: *unc-119(ed4)*; *syEx974* [CherryPOPFOP (described below), *unc-11(+)*], PS5628: *syIs197* [Int HS::*lin-3C*, *myo-2*::dsRed, *pha-1*(+), KS(+)], PS5667: *dbl-1(wk70)*; *sem-5(n1779)*, PS5869: *syIs145*; *syIs197*, PS5870: *syIs145 lin-31(n301)*; *syIs197*, PS5872: *syIs145*, *lin-1(n1790gf)*; *syIs197*, PS5879: *dbl-1(wk70); sem-5(n1779)*, PS5881: *lin-17(n698)*; *syIs188*, PS5889: *sem-5(n1779)*, PS5896: *lin-44(n2111)*; *syIs188*, PS5905: *let-23(sy97) syIs145*, PS5906: *let-23(sy1)*; *dbl-1(wk70)*.

PS4807 contains the *ceh-13*::GFP integrated transgene *syIs145* that was obtained by microinjection of pMF1 (Stoyanov et al., 2003) at 10 ng/µL, pBS at 20 ng/µL and *unc-119*(+) at 40 ng/µL into *unc-119(ed4)*; *him-5(e1490)* mutant animals. POPTOP and

POPFOP were previously described in Green et al. (2008). The control construct, POPFOP, is identical to POPTOP but contains mutated TCF binding sites.

Analysis of strains carrying the *ceh-13*::GFP, POPTOP and POPFOP integrated transgenes

GFP and mCherry expression were analyzed using Nomarski optics and fluorescence microscopy. GFP expression was viewed using a Chroma Technology High Q FITC filter set, while mCherry expression was viewed using a Texas Red Filter. Still images were captured with a Hamamatsu digital camera and Improvision Openlab software version 5.02.

ceh-13::GFP, POPTOP and POPFOP expression were scored in the mid-L3 stage when the B.a progeny had moved into their final positions. In all animals examined, POPFOP expression was not observed in the γ cell, indicating that POPTOP can be used as a readout of Wnt signaling activity. The mean pixel intensity of POPTOP expression in the γ cell in each animal was analyzed using the Improvision Openlab under the following conditions: 0.5 sec exposure, contrast set to zero.

Laser Ablations

U and F cell ablations were performed as previously described (Chamberlin and Sternberg, 1993).

Heat-shock induction of HS::*lin-3* transgene

Plates with well-fed animals were sealed with parafilm and floated in a 33°C water bath for 1 hour to induce the heat-shock response. Animals were scored 3 to 6 hours later.

Results

EGF-Ras signaling upregulates transcription of *ceh-13/labial/Hox1* in γ

To study *ceh-13/Hox* regulation by EGF/Ras signaling, we utilized an integrated transcriptional GFP reporter, *syIs145*, that contains about 8 kb upstream sequence and the first and second exon of *ceh-13* fused to GFP. In s*yIs145* males, *ceh-13*::GFP was observed in γ in 100% of animals by the mid-L3 stage (Fig. 2A-B, Table 1A). First, we ablated the U and F male-specific blast cells which are required for proper γ fate specification and express the *lin-3/EGF* ligand (Chamberlin and Sternberg, 1993; Hwang and Sternberg, 2004). In the majority of males in which the U and F cells were killed, we found that *ceh-13*::GFP was absent in γ (Table 1A). Because null alleles of EGF signaling mutants cause larval lethality (Clark et al., 1992; Herman, 1978; Rogalski et al., 1982), we used *let-23/EGFR*, *let-60/Ras* and *sem-5/Grb-2* single reduction-of-function (rf) mutations to determine if EGF signaling is required for *ceh-13* expression. We observed a significant decrease of *ceh-13*::GFP expression in γ in all strains (Fig. 2C-D, Table 1A). Therefore, EGF/Ras signaling upregulates *ceh-13* transcription in γ.

Since EGF/Ras signaling has been shown previously to be sufficient to induce a δ-to-γ fate transformation, we hypothesized that increased EGF signaling would cause ectopic expression of *ceh-13*::GFP in δ. We tested this hypothesis using several different methods. One method was to use a transgenic construct that places the *lin-3/EGF* cDNA under control of a heat-shock promoter to generate ectopic expression of *lin-3/EGF* (Van Buskirk and Sternberg, 2007). We found that 60% of heat-shock treated animals carrying the HS::LIN-3C construct had abnormal *ceh-13*::GFP expression in δ (Fig. 2E-F, Table 1B). We also made use of a *let-60* gain-of-function (gf) allele, *n1046*, which

constitutively activates Ras signaling. We found that in 18% of *let-60(n1046)* animals, *ceh-13*::GFP was ectopically expressed in δ (Table 1B). In addition, a loss-of-function (lf) mutation in the *lin-15* locus, which normally acts to antagonize the EGF/Ras pathway (Clark et al., 1994; Huang et al., 1994), caused *ceh-13*::GFP expression in δ (Table 1B). Our results suggest that increased EGF signaling is capable of promoting *ceh-13*::GFP expression in δ and that *ceh-13*::GFP expression is an early indicator of the γ cell fate.

lin-1/ETS and _lin-31/Forkhead_ function during γ specification

Since we had found that *ceh-13* transcription is controlled by EGF signaling, we investigated whether *lin-1/ETS* and *lin-31/Forkhead*, transcription factors known to mediate other EGF-Ras signaling events such as vulval development (Beitel et al., 1995; Miller et al., 1993; Tan et al., 1998), also regulate *ceh-13* expression. A role for either transcription factor during γ specification has not previously been identified.

lin-1/ETS has both a positive and negative role in γ specification

Members of the ETS domain transcription factor family effect Ras signaling in many organisms (Wasylyk et al., 1998). *lin-1* is the *C. elegans* ETS homolog and has both a positive and a negative role downstream of EGF-Ras signaling in vulval development, excretory duct cell specification, P12 specification and hook development (Beitel et al., 1995; Howard and Sundaram, 2002; Tiensuu et al., 2005). Several results suggest that *lin-1* functions in a similar manner during γ specification. First, we observed a loss of *ceh-13*::GFP expression in γ in both *lin-1(null)* animals and *lin-1(gf)* mutants, indicating that LIN-1 has both positive and negative effects on *ceh-13/Hox1* expression in

γ (Table 2). In addition, we observed ectopic expression of *ceh-13*::GFP in δ, which suggests that LIN-1 inhibits δ from expressing the γ fate (Table 2). Therefore, LIN-1 positively and negatively regulates transcription of *ceh-13*. The requirement of *lin-1* during γ fate specification appears to be minor and may be redundant with other factors because the γ lineage is normal in all *lin-1(e1777null)* animals observed (n=7, H. Chamberlin, unpublished data). In addition, lineage analysis of *lin-1(e1777null)* animals indicated that δ acquires a γ-like fate in six of seven animals, suggesting that *lin-1* acts to inhibit δ from expressing the γ fate. These results also support the use of *ceh-13*::GFP as an indicator of the γ fate.

To confirm that *lin-1* lies downstream of the EGF signal in γ, we tested whether a *lin-1(gf)* mutation could suppress the effects of increased EGF signaling. We found that *ceh-13*::GFP expression in heat-shocked *lin-1(n1790gf)*; HS::EGF animals was similar to *lin-1(n1790gf)* single mutants (Table 2), indicating epistasis of *lin-1* over excessive LIN-3/EGF. Therefore, *lin-1* lies downstream of the EGF pathway and EGF signaling downregulates *lin-1* inhibition of the γ fate in the presumptive γ. *lin-1* also acts to inhibit δ from expressing the γ fate.

<u>*lin-31/Forkhead* upregulates *ceh-13/Hox* expression</u>

lin-31 belongs to the Forkhead family of transcription factors that also acts positively and negatively downstream of the EGF-Ras pathway in vulval development, similar to *lin-1/ETS* (Miller et al., 1993). However, unlike *lin-1/ETS*, *lin-31/Forkhead* was reported to be specific to EGF/Ras signaling during vulval development and was not thought to act during the specification of the B equivalence groups (Tan et al., 1998). We

also found that *lin-31* is not required for hook or P12 fate specification (n=7 and n=32, respectively). However, we found that LIN-31 is required to upregulate *ceh-13* transcription: *ceh-13*::GFP expression in γ was absent in about 36% of *lin-31(bx31*) and 12% of *lin-31(n301)* (Table 2). *n301* is a null allele of *lin-31* (Miller et al., 2000), while *bx31* is presumably a null allele of *lin-31* (Baird and Ellazar, 1999). Since we never observed abnormal *ceh-13*::GFP expression in δ in *lin-31* mutants, it appears that *lin-31* only has a positive role during γ specification. Similar to *lin-1*, *lin-31* also lies downstream of the EGF signal because *lin-31(n301)* is able to suppress the effects of increased EGF signaling due to ectopic expression of the EGF ligand (Table 2). Therefore, *lin-31* is not a vulval-specific effector of EGF/Ras signaling.

In about 90% of *lin-31(bx31lf)* (n=30) and *lin-31(n301lf)* (n=33) mutants, which had wild-type *ceh-13* expression, we observed that γ divided along a transverse axis, similar to δ, rather than along a longitudinal axis (For both strains: $p<0.0001$, Fisher's Exact test). The division plane of γ in *lin-31/lf* mutants strongly resembles that of δ, distinct from the abnormal spindle orientation defects observed in Wnt mutants (discussed in the following section), indicating that effects on the axis of division in *lin-31/lf* mutants are probably caused by fate specification defects. LIN-31 likely regulates other target genes, besides *ceh-13*, that specify γ fate.

<u>*Other transcription factors tested*</u>

A number of other transcription factors have been shown to act downstream or in parallel to the EGF-Ras pathway in *C. elegans* during one or more of the following events: vulval development, P12 specification and larval viability. Mutations in these

factors cause phenotypes similar to those caused by mutations in components of the EGF signaling pathway. These factors include *lin-39/SexcombsReduced/Hox5* (Eisenmann et al., 1998)*, egl-5/Antennapedia/Ultrabithorax/Hox6/8* (Jiang and Sternberg, 1998), *eor-1/PromyelocyticLeukemiaZincFinger* (Howard and Sundaram, 2002), *eor-2* (a novel protein containing potential nuclear localization signals), *sur-2* (a component of the Mediator complex) (Singh and Han, 1995) and *lin-25*, a novel transcription factor that appears to act with *sur-2* (Nilsson et al., 2000). The last four transcription factors have been shown to act together to positively regulate Ras signaling.

Our results suggest that *egl-5*, *lin-39*, *eor-1* and *eor-2* are probably not required for γ expression of *ceh-13*::GFP (Supplemental Table S1). However, we observed that in about 25% of *eor-1(lf)* and *eor-2(rf)* single mutants, *ceh-13*::GFP was expressed several hours earlier than in the wild type, suggesting that *eor-1* and *eor-2* act to negatively regulate γ fate specification. The RNAi results for *egl-5* and *lin-39* are not conclusive since RNAi may only partially reduce gene activity. We did not test the effects of *lin-25* on *ceh-13*::GFP, but (Nilsson et al., 2000) reported that the γ lineage in *lin-25(ar90null)* mutants is intermediate between wild-type γ and δ lineages.

Wnt signaling controls spindle orientation of γ division

As Wnt signaling has been shown to act together with EGF signaling to specify vulval fates and P12 fate by regulating the Hox genes (Eisenmann et al., 1998; Jiang and Sternberg, 1998), we decided to test whether the Wnt signaling pathway also specified the γ fate. There are five Wnt-like genes in the *C. elegans* genome — *lin-44*, *egl-20*, *mom-2*, *cwn-1* and *cwn-2* — and we examined *ceh-13*::GFP expression in Wnt mutants.

None of the Wnt single or double mutants displayed defects in *ceh-13*::GFP expression (Table 3). However, we observed abnormal mitotic spindle orientation of γ in *mom-2(lf)* homozygotes derived from *mom-2(lf)/+* hermaphrodites and *lin-44(lf)* males. Specifically, in 44% of *lin-44(n2111lf)* and 22.7% of *mom-2(or42lf)* males, γ divided along a more transverse axis than a wild-type longitudinal axis (Fig. 3, Table 3). Because *lin-17/Fz* has been shown to act downstream of *lin-44/Wnt* earlier in the B lineage as well as during other developmental events, we tested if *lin-17(n698rf)* males had similar γ defects. We found that γ divided transversely in 27% of *lin-17(rf)* males, while *ceh-13::*GFP expression was wild-type in all mutants (Table 3). *lin-44(lf)* and *lin-17(rf)* mutants were also abnormal in that the division axis of γ in some animals was almost perpendicular to the wild-type axis: the posterior daughter was slightly dorsal in relation to the the anterior daughter instead of the opposite (as in the wild type). The *mom-2(or42)* defect was not as severe as *lin-44(lf)* or *lin-17(rf)*: γ division was more transverse in *mom-2(or42)* males than in wild-type males, but the angle of division was oblique. Therefore, Wnt signaling involving *lin-44*, *mom-2* and *lin-17* is probably required to orient the γ mitotic spindle but is not required for γ fate specification (based on *ceh-13* expression).

Because *ceh-13* expression is only one marker of γ fate, we next used another criteria of γ fate specification, the number of progeny generated, to determine if Wnt signaling is required to specify fate in addition to orienting the γ mitotic spindle. Since *lin-44(lf)* mutants had the most penetrant γ defect, we performed lineage analysis of γ in *lin-44(lf)* males in which γ divided in a δ-like orientation. We observed that γ divided more than once in all six *lin-44(lf)* males in which γ divided transversely, indicating that

the *lin-44* mutation does not cause a true γ-to-δ fate transformation. Thus, *lin-44* appears to only be required for γ spindle orientation and not γ fate specification. Since the *mom-2(lf)* and *lin-17(rf)* animals we examined have less severe or less penetrant defects than *lin-44(lf)* animals, it is unlikely that they will have a more severe lineage defect (i.e. fewer progeny) than *lin-44(lf)* animals.

Wnt signaling has been shown to orient the mitotic spindle of the EMS and ABar blastomeres in the *C. elegans* embryo without requiring transcriptional activity (Schlesinger et al., 1999; Walston et al., 2004). To determine if *lin-44/Wnt* and *lin-17/Fz* act through *pop-1/TCF* to regulate the transcription of target genes and influence γ spindle orientation, we investigated the expression of POPTOP (a fluorescent reporter containing seven copies of the TCF binding site) (Green et al., 2008) in *lin44(lf)* and *lin-17(rf)* mutants. The mean pixel intensity of POPTOP expression was lower, but not significantly, in *lin-44(lf)* and *lin-17(rf)* males as compared to expression in wild-type males (Fig. 4). Our results suggest that *lin-44* and *lin-17* regulate spindle orientation without requiring transcription in γ. In addition, the lack of effect on target gene transcription supports the *ceh-13* expression assay and lineage analysis that *lin-44* and *lin-17* are not involved in γ fate specification. POPTOP expression in δ indicated that Wnt signaling is involved in δ specification as well.

TGF-β pathway acts either downstream or in parallel to EGF signaling during γ fate specification

We have shown that similar to vulva, hook and P12 specification, EGF and Wnt signaling act together to influence γ development. We have shown that *ceh-13*

expression responds to EGF signaling, which specifies the γ fate. Previously, TGF-β signaling was reported to play a role in γ specification: Stoyanov et al. (2003) reported that mutations in the TGF-β signaling components *dbl-1/ dpp/TGF-β*, *sma-2/R-Smad*, *sma-3/R-Smad* and *sma-4/Co-Smad*, caused loss of *ceh-13*::GFP expression in γ. We wished to further investigate the role of TGF-β signaling in γ specification. *wk70* is a null allele of *dbl-1* which truncates the mature domain (Suzuki et al., 1999). First, we confirmed the findings of Stoyanov et al. (2003) that *ceh-13* expression in γ was abolished in *dbl-1(wk70)* males (n=14). We also observed that in 2 of 4 animals, γ divided in a wild-type longitudinal direction, indicating that γ fate specification was not completely defective in *dbl-1(wk70)* males. This result suggests that other signaling pathways, such as the EGF pathway, likely act with DBL-1 to specify γ fate.

Next, to determine whether the EGF pathway acted downstream of the TGF-β pathway, we investigated whether EGF signaling was sufficient to specify the γ fate when TGF-β activity was reduced. Therefore, we tested whether increased EGF signaling was sufficient to induce *ceh-13*::GFP expression in a *dbl-1(null)* background because increased EGF signaling was sufficient to induce a δ-to-γ fate transformation (Chamberlin and Sternberg, 1994). We found that there was a loss of *ceh-13*::GFP expression in γ in all 13 heatshocked HS::EGF; *dbl-1(null)* males examined. Our results indicate that signaling by the TGF-β ligand *dbl-1* acts either downstream or in parallel to the EGF pathway to specify the γ fate.

TGF-β signaling does not appear to be required for VPC and P12 fate specification

Since EGF signaling plays a major role during γ fate specification, we decided to investigate if TGF-β signaling was also required in other specification events in which the EGF pathway was the major inductive signal. If TGF-β signaling acts only during γ specification, it may contribute to the specificity of γ cell fate versus the other cell fates that require EGF signaling. Although *dbl-1(wk70)* animals exhibit wild-type vulval and P12 development (Table 4), it is possible that *dbl-1* may only play a minor role in these specification events that is revealed in a sensitized background. Therefore, we next tested whether reduced TGF-β signaling would enhance the vulval and P12 defects caused by reduced EGF activity to determine whether *dbl-1/TGF-β* was required during VPC and P12 specification. Because *let-23(null)* mutations cause larval lethality, we constructed double mutants of *dbl-1(wk70)* with *let-23(rf)* or *sem-5(rf)* alleles. *sy1* is a weak rf allele of *let-23* that causes vulval induction defects but no P12 defect (Aroian and Sternberg, 1991). *sy97* is a severe rf allele of *let-23* that causes a completely penetrant Vul phenotype and a partially penetrant P12-to-11 transformation (Aroian and Sternberg, 1991; Jiang and Sternberg, 1998). *n1779* is a weak rf allele of *sem-5* that was reported previously to cause a slight Vul phenotype (Clark et al., 1992). We found that vulval defects in *let-23(sy1)*; *dbl-1(wk70)* and *sem-5(n1779)*; *dbl-1(wk70)* double mutants were similar to *let-23(sy1)* and *sem-5(n1779)* single mutants, respectively (Table 4). These results suggest that *dbl-1* is not required for vulval induction.

We were unable to determine if *dbl-1(wk70)* could enhance the P12 defects observed in *let-23(sy97)* animals because *let-23(sy97)*; *dbl-1(wk70)* animals were embryonic lethal. Therefore, we examined P12 fate in *let-23(sy1)*; *dbl-1(wk70)* and *sem-*

5(n1779); *dbl-1(wk70)* double mutants because although *let-23(sy1)* and *sem-5(n1779)* animals have no P12 defects, they may still provide a sensitized background in which EGF signaling is reduced in P12. Our results suggest that *dbl-1* does not act during P12 development, as we observed a wild-type P12 in 100% of double mutants (Table 4). However, *sy1* and *n1779* are hypormophic mutations, and it is possible that they do not sufficiently affect the functioning of their gene product during P12 specification.

Discussion

We have demonstrated that the EGF and Wnt pathways act together during γ development in the male but each pathway performs different roles. EGF signaling upregulates a Hox gene, *ceh-13/labial*, in γ. This is similar to vulval development and P12 specification, in which EGF signaling upregulates the Hox genes *lin-39/Scr* and *egl-5/Ant/Ubx*, respectively. Wnt signaling helps orient the γ mitotic spindle but does not appear to be required for γ specification. Single or double Wnt mutants did not have defects in *ceh-13/labial* expression, but *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* mutants had defects in orienting the γ mitotic spindle. Finally, we have shown that TGF-β signaling by the *C. elegans dpp* ortholog *dbl-1* likely acts in γ fate specification and not in VPC induction or P12 specification (i.e., other EGF and Wnt regulated developmental events).

EGF and Wnt signaling roles during γ development

EGF-Ras signaling has previously been shown to specify the γ fate, and we showed that *ceh-13/labial* is transcriptionally regulated by EGF-Ras signaling in γ. In addition, we found that the transcription factors *lin-1/ETS* and *lin-31/Forkhead* play a role during γ specification. It has been suggested that *lin-31* acted only during vulval development downstream of EGF-Ras signaling (Tan et al., 1998). However, our results indicated otherwise, and *lin-31/Forkhead* did not appear to confer specificity to EGF-Ras regulated fate specification events in *C. elegans*. TGF-β signaling has been previously reported to be absolutely required for *ceh-13* expression, indicating a role for TGF-β during γ fate specification. We confirmed those results but also observed that in some

dbl-1(null) males, γ displays a wild-type axis of division. We also demonstrated that signaling by DBL-1 probably acts downstream or in parallel to the EGF pathway to specify γ fate.

All Wnt single or double mutants examined had wild-type *ceh-13/labial* expression in γ. Because there are five Wnt genes in *C. elegans*, we were unable to definitively rule out a role for Wnt signaling in regulating *ceh-13/labial* expression. However, γ divided in a δ-like manner (transverse) in *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* mutants. Furthermore, *lin-44* and *lin-17* spindle defects were more severe than in *mom-2*: the axis of division was sometimes almost perpendicular to the wild-type axis. In six *lin-44(lf)* males in which γ divided along a transverse axis, γ divided more than once (characteristic of the γ fate), indicating that γ did not undergo a true γ-to-δ transformation in *lin-44/Wnt* mutants. In addition, the effects of *lin-44* on γ division did not appear to require gene expression, based on our analysis of Wnt reporter expression (POPTOP). *lin-17(rf)* males had similar POPTOP expression as *lin-44(lf)* males, suggesting that *lin-17* effects are similar to *lin-44*. Our data suggest that Wnt signaling by *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* is required to orient the γ mitotic spindle, and that *lin-44/Wnt* and *lin-17/Fz* function mainly through a non-transcriptional mechanism. We do not have evidence that *lin-44/Wnt*, *mom-2/Wnt* and *lin-17/Fz* are required to specify other aspects of γ fate.

Because LIN-44 and LIN-17 are required to specify B fate (Herman and Horvitz, 1994; Sawa et al., 1996; Sternberg and Horvitz, 1988), we bypassed their requirement earlier in the lineage by using a *lin-17* reduction-of-function allele. It was extremely difficult to find *lin-17(n671lf)* males that had wild-type B cell specification so that we

could determine γ defects. By comparison, although *lin-44(n2111)* has been described as a null allele (Herman and Horvitz, 1994), we were able to find enough males in which B divided and produced a γ/δ pair. A different null allele of *lin-44*, *n1792*, had very few males that had wild-type B specification, suggesting that there was still some gene function in *n2111* mutants. Similarly, *mom-2(lf)* homozygotes may still have some MOM-2 activity because MOM-2 is required maternally during embryogenesis and *mom-2lf)* homozygotes examined were derived from *mom-2/+* hermaphrodites. Therefore, we cannot exclude the possibility that sufficient gene function in each of the Wnt signaling mutants may have masked a requirement during fate specification based on our assays (i.e., number of progeny generated, *ceh-13* and POPTOP expression).

It is also possible that the Wnt pathway plays a role in γ fate which will be revealed upon reducing the activity of the right combination of Wnts, since multiple Wnts have been shown to act redundantly during other *C. elegans* developmental events and POPTOP is expressed in γ (Gleason et al., 2006). Alternatively, similar to the ABar blastomere, Wnt transcriptional activity is required to maintain proper timing of the spindle rotation (Walston et al., 2004). Therefore, other roles for Wnt signaling during γ fate specification remain to be uncovered.

We propose that EGF and TGF-β activity specify γ by controlling target gene expression, while Wnt signaling acts to orient the γ mitotic spindle without requiring transcriptional activity (Fig. 5A). Since the axis of division of γ in rf mutants of components of the EGF pathway are mostly either γ-like (longitudinal) or δ-like (transverse), EGF signaling probably controls spindle orientation as a consequence of specifying the γ fate and does not directly target the cytoskeleton.

Comparing EGF and Wnt regulated Equivalence groups

Comparing the VPCs, HCG, P11/12 and γ/δ equivalence groups allows us to identify several similarities and differences that may explain how the same signaling pathways specify different fates in different equivalence groups. First, we have found another example where EGF/Ras signaling controls a Hox gene during fate specification in *C. elegans* (Fig. 5B). Although a role for *ceh-13/labial/Hox* in γ fate specification was not found (data not shown), we cannot rule out a requirement for *ceh-13* because we were unable to assay terminal fates. Moreover, the upregulation of *ceh-13/labial* by EGF signaling, which specifies γ fate, and the conservation of Hox function in other cell fates regulated by EGF and Wnt signaling hints at a functional role for *ceh-13* in γ. EGF and/or Wnt signaling upregulate *lin-39/Scr/Hox* to specify VPC fate (Eisenmann et al., 1998), *egl-5/Abd-B/Hox9-13* to specify P12 fate, and *mab-5/ftz/Hox* during hook development (see Intro). Alternatively, *ceh-13* may play a lesser role during fate specification

One reason why *ceh-13*, as opposed to the other Hox genes, is upregulated in γ may be due to TGF-β signaling, which also regulates *ceh-13* expression and specifies γ fate. Since the TGF-β signaling pathway does not appear to be involved in vulval and P12 specification, it probably does not act to regulate Hox genes in the VPCs and P11/12.

Another possibility is that the specificity of Hox expression in the different equivalence groups may be a consequence of their developmental history. Prior to upregulation by EGF and/or Wnt signaling, *lin-39* and *mab-5* are already expressed in the VPCs and HCG, respectively. One possibility is that the presence of a different Hox gene in these two equivalence groups may bias the VPCs and the HCG to upregulate *lin-*

39 and *mab-5*, respectively, in response to EGF and/or Wnt signaling. In the case of the γ/δ equivalence group, there is no prior expression of *ceh-13* in either cell within the equivalence group. *egl-5* is most probably not expressed in P11/12 before specification (Ferreira et al., 1999).

A third potential explanation for why different Hox genes are upregulated in each equivalence group is the relative importance of each pathway for specification (possibly a function of the distance from the source of each ligand) within each equivalence groups. During vulval development, EGF signaling is the major inductive pathway, whereas Wnt signaling is the major inductive pathway during hook development. By comparison, EGF and Wnt signaling appear to contribute equally to P12 specification, while Wnt plays a major role in inducing hook development and a requirement for EGF signaling is only seen when Wnt activity is reduced. Our results support a role for EGF and TGF-β signaling, but not Wnt signaling, in γ fate specification. The different levels of signaling activity different equivalence groups by each pathway in specifying may result in the upregulation of a different Hox gene.

In contrast to the other equivalence groups, patterning of the γ/δ equivalence pair appears to involve competing signals from different cells outside the equivalence group to specify the γ and δ fates. Both fates are specified by other cells and do not appear to be required to specify each other. Therefore, there is no primary (1°) fate in the γ/δ equivalence group: isolated γ/δ precursors can adopt either the γ or δ fate (Chamberlin and Sternberg, 1993; Sulston and White, 1980). In contrast, VPC and HCG specification utilize a sequential signaling mechanism to first specify the 1° fate, followed by lateral signaling to specify the 2° fate. Specification of the 2° fate usually requires the presence

of the 1° fate. However, a graded signaling mechanism in which the EGF signal acts to specify the 1° and 2° VPC fates allows for isolated 2° fates. In general, however, the VPCs and HCG, the same signals from the same cells to specify both the 1° and 2° fates. The P12 fate is the 1° fate within the P11/12 pair because an isolated P11/12 precursor always adopts the P12 fate, suggesting that there is no competing P11 fate specification signal. A sequential signaling mechanism does not appear to be used to specify the P11 fate, and there is no evidence for a model in which competing signals act to specify the P11 and P12 fates. Although the source of the EGF and Wnt patterning signals have not been determined for P12 specification, reduced EGF or Wnt activity results in the P11/12 pair adopting the P11 fate and intermediate P11/12 fates have not been observed. Neither a P11 fate specification signal nor a cell that specifies P11 fate has been identified.

Since several competing external signals specify the γ/δ pair and the axis of division of each fate in the γ/δ pair is distinct (transverse versus longitudinal), we were able to observe that fate specification and mitotic spindle orientation of γ appear to be separable. This is similar to EMS blastomere development where orientation of the EMS mitotic spindle (by a non-transcriptional mechanism) and endoderm fate induction (by regulating gene transcription) are regulated by different Wnt subpathways (Rocheleau et al., 1997; Schlesinger et al., 1999). Within the γ/δ pair, Wnt signaling acts to orient the γ mitotic spindle but does not seem required for fate specification. By comparison, mitotic spindle defects are not observed in the other EGF and Wnt specified fates, P6.p (1° VPC), P11.p (1° HCG) and P12, when EGF and/or Wnt signaling is compromised because the fate acquired by these cells either has the same mitotic spindle orientation or does not involve division. For example, the 3° VPC fate adopted by P6.p in *bar-1/β-*

catenin mutants results in P6.p dividing once along the same axis that it would have divided if it had adopted the 1° fate. Further study of each equivalence group will allow us to determine other generalities of how the same signals are used to specify different cell fates and to determine how the same signals interact differently to specify fate.

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Tables

Table 1A. Reduced EGF signaling causes loss of *ceh-13*::GFP expression

Genotype[a]	n	*ceh-13*::GFP[b] in γ (%)
Intact, wild type	41	100
Mock ablated, wild type	3	100
$U^{-}F^{-}$ [c]	8	12.5***
let-60(rf)/Ras	42	57.1***
let-23(rf)/EGFR[d]	20	55***
sem-5(rf)/Grb-2	30	26.7***

***$p<0.0001$

[a] The alleles used were *let-23(sy97)*, *let-60(n2021)* and *sem-5(n1619)*. All strains contained *him-5(e1490)*.

[b] All strains examined carried the integrated *ceh-13*::GFP transgene, *syIs145*.

[c] F and U were ablated in these animals.

[d] *ceh-13*::GFP expression was much dimmer than wild-type expression in 7 of the 11 *let-23(rf)* males that had expression in γ.

Table 1B. Increased EGF signaling causes ectopic *ceh-13*::GFP expression

Genotype[a]	n	*ceh-13*::GFP[b] in δ (%)
Wild type	41	0
Wild type, 1 hr heat-shock	25	0
lin-15(lf)	38	18.4**
Integrated HS::EGF, 1 hr heat-shock	30	86.7***
let-60(gf)/Ras	28	17.9*

***$p<0.0001$
**$p<0.005$
*$p<0.05$
[a] The alleles used were *lin-15(e1763)* and *let-60(n1046)*. The integrated HS::EGF transgene *syIs197* was used. All strains contained *him-5(e1490)*.
[b] The integrated *ceh-13*::GFP transgene *syIs145* was used.

Table 2. *lin-1* and *lin-31* regulate *ceh-13*::GFP expression

Genotype[a]	**n**	***ceh-13*::GFP**[b] **in γ (%)**	***ceh-13*::GFP in δ (%)**
Wild type	41	100	0
lin-1(e1777lf)	34	85.3*	41.2***
lin-1(n1761gf)	30	76.7**	0
lin-1(n1790gf)	30	30***	0
lin-31(bx31lf)	33	63.6***	0
lin-31(n301lf)	32	87.5*	0
Int HS::*lin-3*[b]	30	100	86.7***
lin-1(n1790gf); Int HS::*lin-3*[b]	15	33.3***	0***
lin-31(n301lf); Int HS::*lin-3*[b]	30	83.3	26.7***

***p<0.0001
**p<0.005
*p<0.05
[a] All strains contained *him-5(e1490)* and the integrated *ceh-13*::GFP transgene *syIs14*5.
[b] The integrated HS::EGF transgene *syIs197* was used.

Table 3. Wnt signaling controls spindle orientation in γ.

Genotype[a]	n	γ division plane
		Abnormal (L/R) (%)
Wild type	30	0
Wnts		
cwn-2(ok895lf)	30	0
egl-20(hu120rf)	27	0
cwn-1(ok546lf); egl-20(n585rf)	33	0
lin-44(n2111lf)	34	44.1***
mom-2(or42lf)	22	22.7*
Wnt receptor		
lin-17(n698rf)	33	27.3**

***$p<0.0001$
**$p<0.005$
*$p=0.01$
[a] All strains contained *him-5(e1490)* and the integrated *ceh-13*::GFP transgene *syIs14*5.

Table 4. *dbl-1/TGF-β* does not appear to be required for VPC or P12 specification

Strains[a]	Vulval Induction Index (n)	% P12→11 transformation (n)
dbl-1(lf)	3.0 (54)	0 (36)
let-23(rf)	0.27 (39)	0 (21)
let-23(rf); *dbl-1(lf)*	0.31 (27)	0 (37)
sem-5(rf)	3.0 (81)	0 (23)
sem-5(rf); *dbl-1(lf)*	3.0 (50)	0 (11)

[a] The alleles used were *dbl-1(wk70)*, *let-23(sy1)* and *sem-5(n1779)*. All strains contain *him-5(e1490)*.

Figures

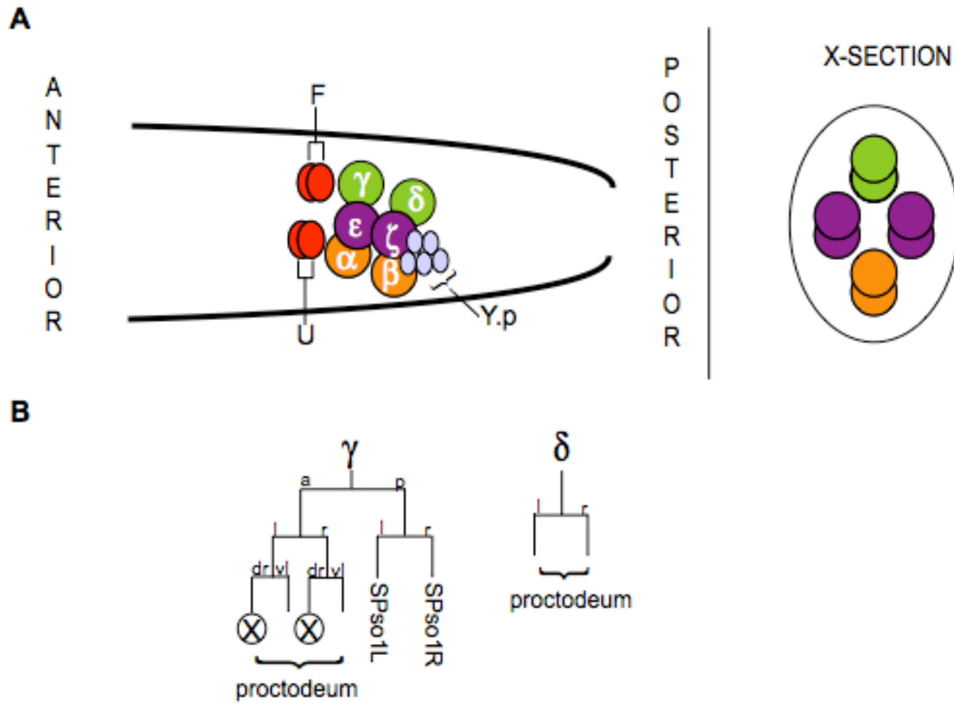

Fig. 1

Figure 1. The γ/δ equivalence group during development. (A) Arrangement of the B progeny during the mid-L3 stage. Left lateral view and cross section. (B) Cell division patterns of γ and δ, adapted from Sulston et al. (1980). Circled crosses indicate pairs of cell in which the left or right cell dies.

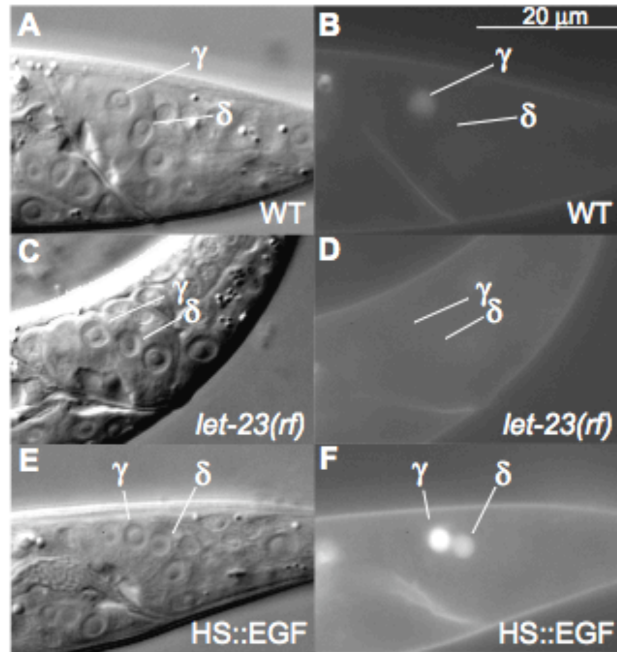

Fig. 2

Figure 2. EGF signaling is necessary and sufficient for *ceh-13*::GFP expression in the γ/δ pair (A-B) Mid-L3. Wild-type *ceh-13*::GFP expression was only observed in γ. (C-D) Mid-L3 *let-23(rf)* males. *ceh-13*::GFP was not expressed in γ. Similar observations were made in *sem-5(rf)* and *let-60(rf)* mutants. (E-F) Mid-L3. Increased EGF signaling in heat-shocked HS::EGF males caused ectopic *ceh-13*::GFP expression in δ, in addition to wild-type γ expression. Similar observations were made in *lin-15(lf)* and *let-60(gf)* mutants. Left lateral views. Scale bar in B, 20 μm for A-F.

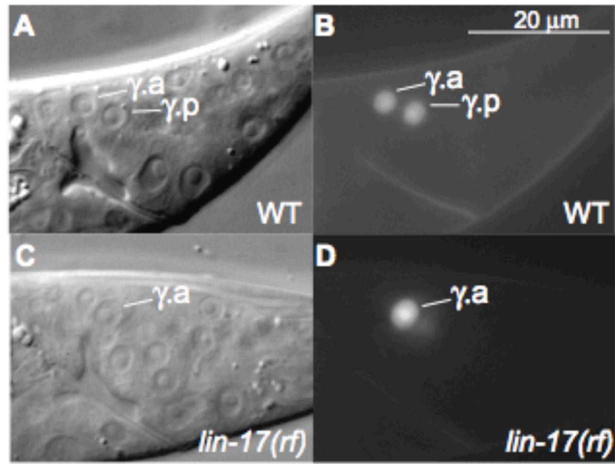

Fig. 3

Figure 3. Wnt signaling is required to orient the γ mitotic spindle. (A-B) Mid-L3. γ divides along longitudinal axis in wild-type males. (C-D) Mid-L3 *lin-17(n698rf)* male. γ divides in a transverse manner. Only γ.a can be seen in this plane and the more posterior daughter of γ is out of focus in this picture. Left lateral views. Scale bar in B, 20 μm for A-D.

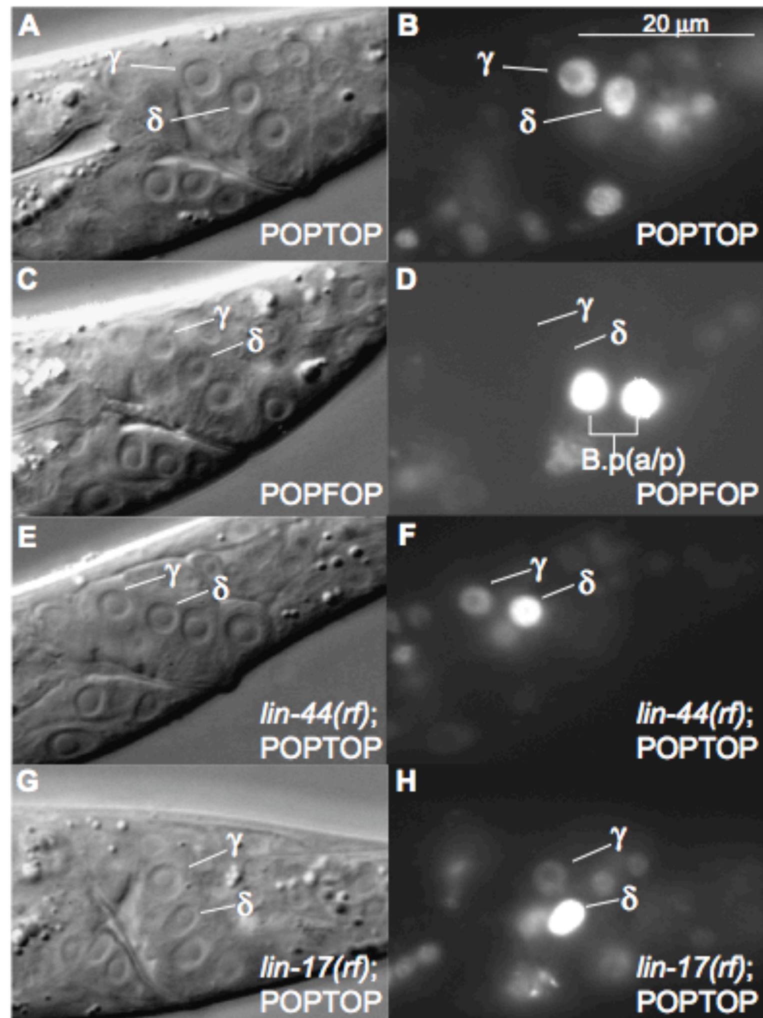

Fig. 4

Figure. 4. *lin-44/Wnt* and *lin-17/Fz* do not appear to be required for POPTOP expression in γ. (A-B) Mid-L3 male. Wild-type POPTOP expression in γ and δ. (C-D) Mid-L3. POPFOP control reporter was not expressed in γ and δ, indicating that POPTOP expression in those cells is due to Wnt activity. (E-F) Mid-L3 *lin-44(lf)* male. POPTOP expression was observed in all *lin-44(lf)* males examined. (G-H) Mid-L3 *lin-17(rf)* male. POPTOP expression was observed in all *lin-17(rf)* males examined. Although pixel count analysis of POPTOP expression in γ indicated that the average expression in *lin-44* and *lin-17* mutants was lower than in wild-type, the difference in expression was not statistically significant. Left lateral views. Scale bar in B, 20 μm for A-F.

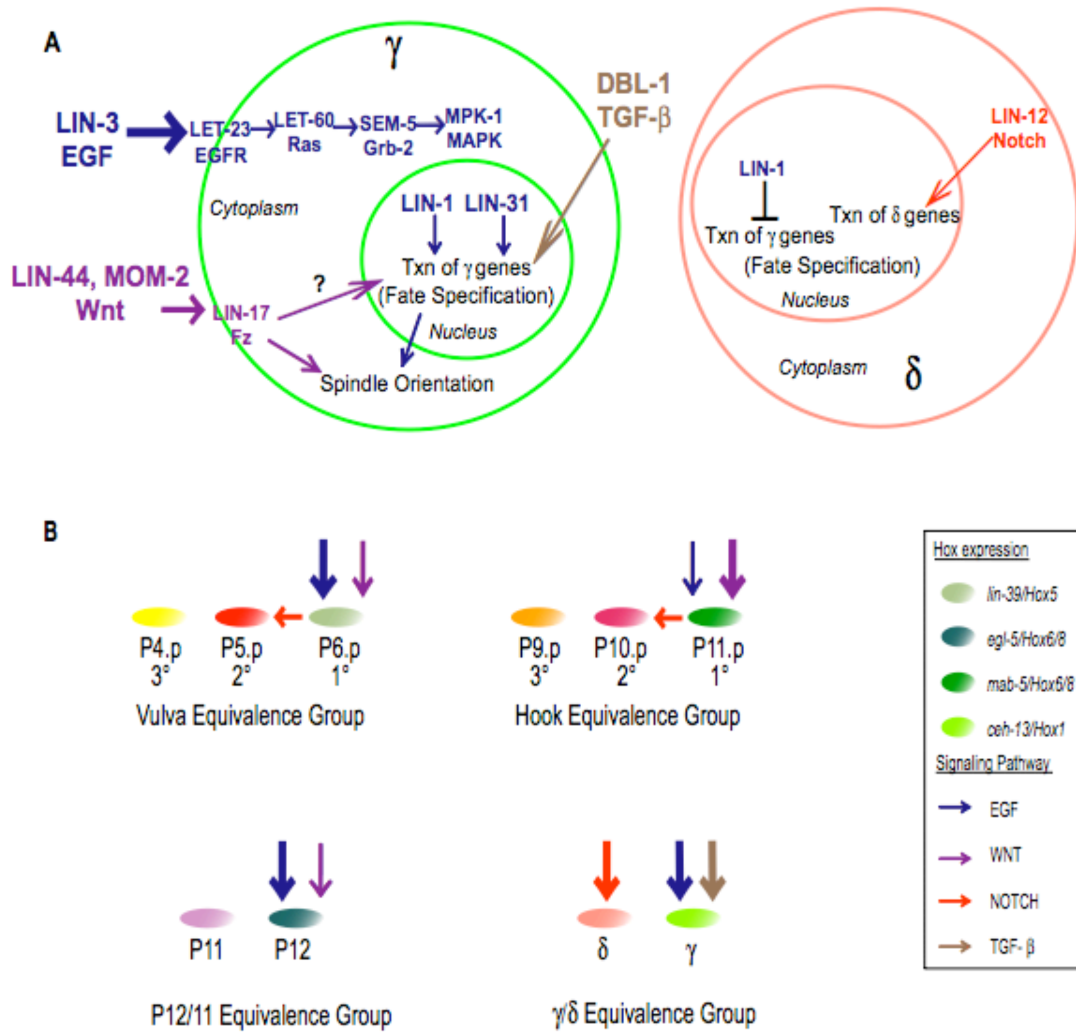

Fig. 5

Figure 5. Patterning of equivalence groups in *C. elegans* (A) Model for EGF, Wnt and TGF-β signaling during γ/δ specification. The EGF and TGF-β pathways specify γ fate by regulating the transcription of target genes such as *ceh-13/hox1*. Wnt acts to orient the mitotic spindle of γ. POPTOP expression suggests Wnt may play a role in γ fate specification. (B) A comparison of the HCG, VPCs, P11/12 and γ/δ groups. EGF and Wnt signaling have different requirements relative to each other during the patterning of each equivalence group. This difference may account for the specificity of fate by both pathways induced in each group. In addition, Wnt signaling orients the mitotic spindle during γ development. Such a role for Wnt signaling has not been observed in the other equivalence groups. Another factor that may contribute to fate specification in each equivalence group is the use of a third pathway during patterning. TGF-β signaling by *dbl-1/dpp* is required to specify γ fate and does not appear to act during VPC and P12 specification, equivalence groups in which EGF signaling is the major inductive signal. Finally, downstream of the EGF and Wnt pathways, a different Hox gene is expressed in each equivalence group and required to specify fate within that group. One exception is *ceh-13/Hox1* for which a functional role in γ fate specification has not been identified.

Supplemental Information

Table S1. Transcription factors that were not required for *ceh-13*::GFP expression

Genotype[a]	n	*ceh-13*::GFP[b] in γ (%)
Intact, wild type	41	100
eor-1(ok1127)[b]	33	100
eor-1(cs28null)	37	2.7
eor-2(cs42rf)	32	6.25
egl-5 RNAi[c]	20	100
lin-39 RNAi[c]	20	100

[a] All strains contained *him-5(e1490)*.
[b] The *ok1127* allele was made by the OMRF Knockout Group and has an estimated deletion of about 1.2 kb.
[c] Feeding RNAi was carried out using clones from the Ahringer Library.

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CHAPTER 4

Concluding Remarks

Summary

I began my thesis work interested in how the same signaling pathways were used during development to specify different fates. EGF and Wnt signaling had been demonstrated to act together with different Hox genes during the specification of two equivalence groups in *C. elegans*, the VPCs and the P11/12 pair (Eisenmann et al., 1998; Jiang and Sternberg, 1998; Wagmaister et al., 2006). Both or one of the EGF and Wnt pathways as well as two other Hox genes, *mab-5/Hox6/8* and *ceh-13/labial/Hox1*, were also implicated in the development of two other equivalence groups, the HCG and the γ/δ pair (Chamberlin and Sternberg, 1994; Sternberg and Horvitz, 1988; Stoyanov et al., 2003) (H. Yu, personal communication). I wanted to investigate the conservation of Hox regulation by EGF and Wnt since one mechanism by which the same signaling pathways specify different fates may be through the regulation of such master control genes. The work I have presented on the patterning of the hook and γ/δ equivalence groups provides further support for the upregulation of Hox genes by EGF and/or Wnt pathway to specify fate in *C. elegans*.

Previous work had indicated that the Wnt receptor *lin-17/Fz* acted during HCG fate execution (H. Yu and P.W. Sternberg, personal communication), but it was unknown if it also acted during HCG fate specification and whether Wnts were involved as the hook inductive signal. In Chapter 2, I determined that Wnt signaling was the major hook inductive signal, and that the Wnts, in particular *lin-44* and *egl-20*, and *lin-17/Fz* are required to specify 1° and 2° HCG fates. A minor role for EGF signaling during hook specification was revealed only when Wnt activity was reduced. In addition, genetic analysis suggested that *mab-5/Hox6/8* functioned downstream of ectopic Wnt signaling to

specify hook fates outside the HCG (H. Yu, personal communication). I showed that Wnt signaling is required for *mab-5/Hox* expression in P11.p, providing the link between the two genes within the HCG. Since P11.p acquires the 1° hook fate and in turn specifies the 2° fate, my data provides further support that *mab-5/Hox6/8* is required to specify HCG fates.

In Chapter 3, I demonstrated that the EGF pathway is both necessary and sufficient to upregulate *ceh-13/labial/Hox1* expression in γ. In addition, I uncovered a role for the transcription factors *lin-1/Ets* and *lin-31/Forkhead* in regulating *ceh-13*::GFP expression and specifying the γ fate. TGF-β signaling had previously been shown to be absolutely required for *ceh-13*::GFP in γ, and I showed that the TGF- β ligand, *dbl-1*, does not act upstream of the EGF pathway to control *ceh-13*::GFP expression. My results indicated that TGF-β signaling either acts downstream or in parallel to EGF signaling during the regulation of *ceh-13*::GFP in γ and hence γ fate specification. I did not find evidence that Wnt signaling specifies the γ fate but found that the Wnts, *lin-44* and *mom-2*, and *lin-17/Fz* are required for γ division along the correct axis without significantly affecting POPTOP expression, suggesting that Wnt signaling orients the γ mitotic spindle probably by a transcription-independent mechanism. Such a function for Wnt signaling had not been observed in the other EGF-regulated *C. elegans* equivalence groups.

If Hox genes confer specificity downstream of EGF and Wnt signaling, how are different Hox genes upregulated in different equivalence groups? The characterization of the HCG and γ/δ equivalence pair provides some clues to how specific Hox genes are upregulated. First, HCG specification is similar to VPC and P12 specification in that

both EGF and Wnt signaling are required. However, Wnt signaling is the major hook inductive signal, and EGF signaling plays a minor role. By comparison, EGF signaling is the major inductive signal during vulval and γ specification. The EGF pathway also plays a more significant role during P12 specification as compared to hook induction. Therefore, the different relative importance of each signaling pathway during the development of each equivalence group might lead to the specificity of Hox gene expression. Second, another signaling pathway, TGF-β, upregulates *ceh-13/Hox* and appears to act only during γ fate specification. Therefore, TGF-β signaling may help to confer specificity to Hox expression in γ.

Where do we go from here?

In *Drosophila*, Hox expression patterns are known to be controlled by the gap and pair-rule genes (Veraksa et al., 2000). However, the upstream mechanisms that generate Hox expression in mammals remain poorly understood. Furthermore, regulation of Hox genes is of great interest because Hox gene expression is altered in a variety of cancers (Nunes et al., 2003). Although the Hox cluster in *C. elegans* does not exhibit spatial colinearity as neatly as in higher organisms, conserved regulatory cis-elements in the *lin-39/ceh-13* subcluster have been identified that drive the same expression pattern between species (Kuntz et al., 2008). Some of these elements are expected to regulate the transcription of both genes, and expression of these elements has not been characterized in the male. Since I have shown that EGF signaling controls the expression of *ceh-13*, in addition to *lin-39*, further analysis of the non-coding regions required for their expression and identification of elements which respond to EGF signaling will lead to a better

understanding of how the EGF pathway generates tissue-specific Hox expression. Similar analysis can be carried out with the *mab-5* and *egl-5* intragenic region.

Another direction to take would be to delve deeper into the mechanism of Hox specificity. There is growing evidence that Hox genes interact with a large variety of transcription factors to specify fate throughout development. Although candidate gene approaches using RNAi in *C. elegans* have proven to be a relatively easy and quick way to screen for factors of interest, RNAi does not seem to be effective in the VPCs, the most well-characterized equivalence group (J. Sanders, personal communication). Recently developed techniques such as single cell RNA sequencing may provide a better way to identify Hox co-factors and target genes in the different equivalence groups.

A third area to explore would be patterning of the other B cell equivalence groups, α/β and ε/ζ. The four Hox genes associated with the VPCs, HCG, P12 and γ fates are not expressed in these two B cell equivalence groups that are regulated by EGF signaling (data not shown). Conveniently, there are two remaining Hox genes in the *C. elegans* cluster that have not been carefully examined in terms of expression and function in the α/β and ε/ζ. These equivalence groups are closely related to the γ/δ pair and are positioned near by, yet they acquire different fates. If they are receiving similar signals, why is *ceh-13* not expressed in the anterior cell (α and ε) of the other two equivalence pairs? Lineage analysis of the B cell equivalence groups is time-consuming and difficult. Cell fate markers and other types of fate assays will be useful in further study of these groups.

To whoever made it to the end of this thesis

For what it's worth, some things I've learned along the way. Don't run to Paul the minute you see something exciting, take a day or at least an hour to think about it first. I still remember the day Cheryl found the two-headed worm…Find someone in lab who you can talk to about your project, besides Paul. If you're lucky, it may turn out to one or all of your labmates who share your room =) Don't believe everything you read. Expression patterns and mutant phenotypes are a few of my favorite things (not!) that have been reported incorrectly in published papers.

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Appendix

Determining *ceh-13/labial/Hox1* function in γ

Because *ceh-13/labial/Hox1* was expressed in γ and EGF signaling regulates γ fate specification, we wanted to test whether *ceh-13* was required for γ fate specification. However, *ceh-13(null)* mutations cause embryonic lethality in the majority of animals and only a small percentage of sickly survivors manage to persist to adulthood. Of these survivors, males do not have any defects in mating, indicating that spicule formation is normal in these mutants (Stoyanov et al., 2003). Because the survivors are not healthy, it is difficult to perform lineage analysis.

To bypass the requirement for *ceh-13/labial* during embryonic development, I made a heat-shock inducible *ceh-13* exon 1 hairpin RNAi construct where the hairpin was cloned into the heat-shock vector pPD49.83. Heat-shock three to five hours before the first γ division had no effect on the axis of division: γ divided longitudinally in all heat-shocked HS::*ceh-13* RNAi animals and *ceh-13*:GFP expression was normal (n=16). Interestingly, I observed that there was ectopic expression of *ceh-13*::GFP in the other B.a progeny in these animals (Appendix Fig. 1): *ceh-13*::GFP was expressed in δ, ζ and α in about 20% of animals and in β and ε in about 50% of animals (n=15). My results suggest that *ceh-13* is present at very low levels to negatively autoregulate its own expression in the other B.a progeny. The effects of HS::*ceh-13* RNAi on α, β, δ, ε and ζ indicate that the construct is able to reduce *ceh-13* levels. However, it is still possible that *ceh-13* activity is not sufficiently lowered by the RNAi construct. Therefore, further analysis will be necessary to determine whether *ceh-13* is required to specify the γ fate.

***mab-5/Antennapedia/Ultrabithorax/Hox6/8* expression in the Male Tail**

mab-5 is expressed in P11.p in early-mid L3 males

I obtained males carrying translational *mab-5*::GFP extrachromosomal arrays from the Waterston lab in which the *mab-5*::GFP construct has GFP immediately inserted prior to the stop codon of *mab-5* within a fosmid clone. I observed *mab-5*::GFP expression in P11.p in all early-mid L3 males examined (n=25, Appendix Fig. 2A-B). I also found that *mab-5*::GFP was expressed in P10.p and P9.p in about half of the animals, although at much lower levels as compared to P11.p. Expression was not observed in any of the B progeny in these animals.

mab-5 expression is controlled by LIN-17/Frizzled

Since I had shown that Wnt signaling through the LIN-17/Fz receptor was required to specify the 1° HCG fate, we decided to test if Wnt signaling was required for *mab-5* expression in P11.p during the L3 stage. I found that only one of eight early L3 *lin-17(n671null)* males had wild-type expression of *mab-5*::GFP. In four males, there was no GFP expression in P11.p, while in the remaining three males, GFP expression in P11.p was very faint (Appendix Fig. 2C-D). Therefore, *lin-17Fz* is required to upregulate *mab-5*::GFP expression in P11.p (Fisher's Exact Test, p=0.0003).

***lag-2/DSL* Expression in the Male Tail**

There are ten DSL coding genes in the *C. elegans* genome (Chen and Greenwald, 2004). I made a transcriptional *lag-2*::YFP reporter containing 6.2 kb of sequence upstream of the start site of the *lag-2* gene. The 6.2kb PCR fragment was designed with

a HindIII site on one end and a BamHI site on the other end and was cloned directionally into the L4643 vector that had been digested with BamH1 and HindIII. The extrachromosomal array carrying this *lag-2*::YFP transgene, *syEx971*, was generated. The integrated line *syIs209* was derived from *syEx971*. I examined *syIs209* males and found that the expression pattern of *lag-2* within the HCG is consistent with a role for Notch signaling in specifying the 2° HCG fate and the Bδ fate. Expression was also observed in ventral cord neurons.

In the HCG

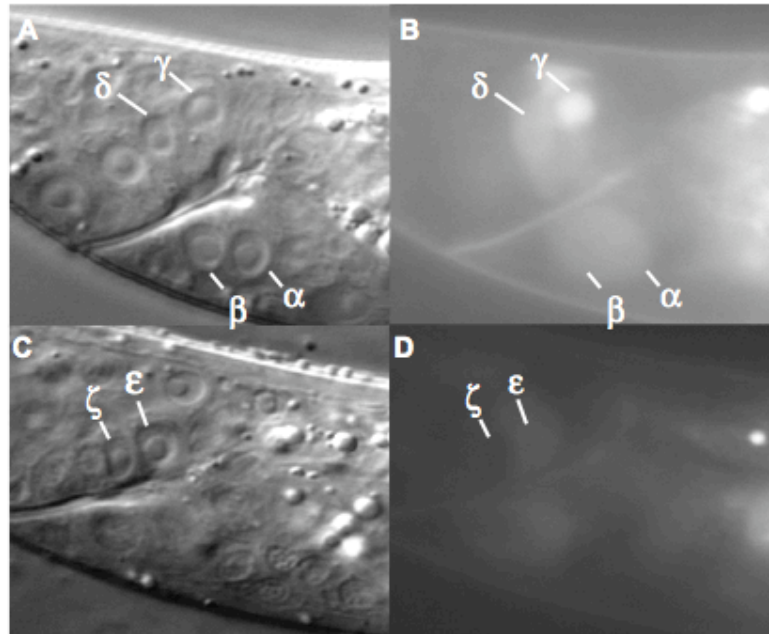
In Chapter 2, we showed that the 1° HCG fate is required to specify the 2° HCG fate, while previous work had indicated that LIN-12/Notch signaling specifies the 2° HCG fate (Greenwald et al., 1983). Consistent with these data, I found that *lag-2* is expressed in the 1°-fated cell P11.p in all early-mid L3 males examined (n=10, Appendix Fig. 3A-B) and in both P11.p daughters in all mid-L3 males examined (n=10). However, I also observed *lag-2::GFP* in P10.p, the presumptive 2° HCG cell, although expression is usually not as bright as in P11.p. One explanation may be that P10.p and P11.p both express *lag-2* prior to fate specification but *lag-2* expression becomes restricted to and upregulated in P11.p starting from the time of hook induction in the L2. The lower levels of expression in P10.p may be residual GFP.

In the B.a progeny

The signal from Y.p and LIN-12/Notch signaling are required to promote the δ fate (Chamberlin and Sternberg, 1994). Because Y.p is not present in *lin-12(null)*

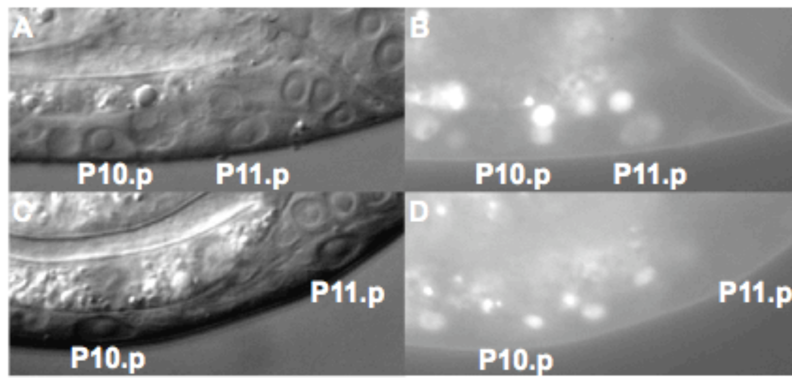
animals, it is not known if Y.p is sufficient to promote the δ fate when Notch signaling is absent. Consistent with a role for Y.p in promoting the δ fate, I found that *lag-2* was expressed in Y.p progeny as well as another cell slightly posterior to the γ/δ pair, likely DVB (Appendix Fig. 3A-B). This raises the possibility that Y.p acts as a major source of the Notch ligand to induce Notch signaling in δ. In addition, I observed *lag-2*::GFP expression in α and β in seven of ten early-mid L3 males.

Appendix Figures



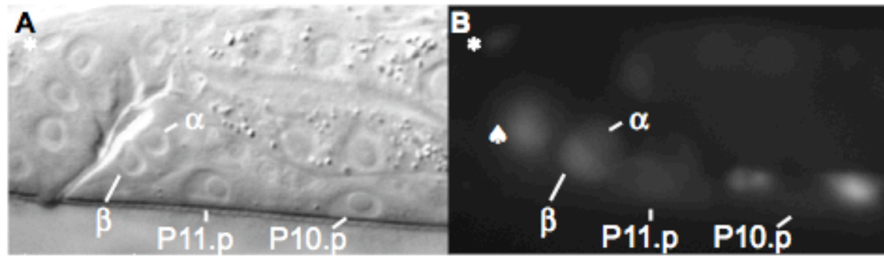
Appendix Fig. 1

Appendix Figure 1. Heat-shock inducible *ceh-13* RNAi caused ectopic *ceh-13*::GFP expression in B.a progeny. (A-B) Mid-L3 male. Ectopic expression was observed in α, β and δ. (C-D) Mid-L3 male. Ectopic expression was observed in ε. Left lateral views.



Appendix Fig. 2

Appendix Figure 2. *lin-17/Fz* is required for *mab-5*::GFP expression in P11.p. (A-B) Mid-L3 male. *mab-5*::GFP was expressed in P10.p at much lower levels than in P11.p. (C-D) Mid-L3 *lin-17(n671null)* male. *mab-5*::GFP was not observed in P11.p, indicating that *lin-17/Fz* upregulates *mab-5* expression. Left lateral views.



Appendix Fig. 3

Appendix Figure 3. *lag-2*::GFP expression in the male tail. (A-B) Mid-L3 male. *lag-2*::GFP was expressed in P11.p, P10.p and the B progeny, α and β. ∗ indicates *lag-2* expressing cell, probably DVB; ♠ indicates expression in Y.p progeny in lateral planes. Right lateral views.

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