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.

lin-31/Forkhead upregulates *ceh-13/Hox* expression

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.

Other transcription factors tested

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.

Acknowledgements

We thank Fritz Muller for pMF1, Cheryl Van Buskirk for the HS::EGF strain, Jennifer Green for the POPTOP and POPFOP integrated lines, Helen Chamberlin for unpublished data, Mihoko Kato and Cheryl Van Buskirk for discussions and helpful comments on the manuscript. P.W.S. is an Investigator of the Howard Hughes Medical Institute. A.S. was supported by a HHMI Pre-Doctoral Fellowship.

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 *syIs145*.

[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 *syIs145*.

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

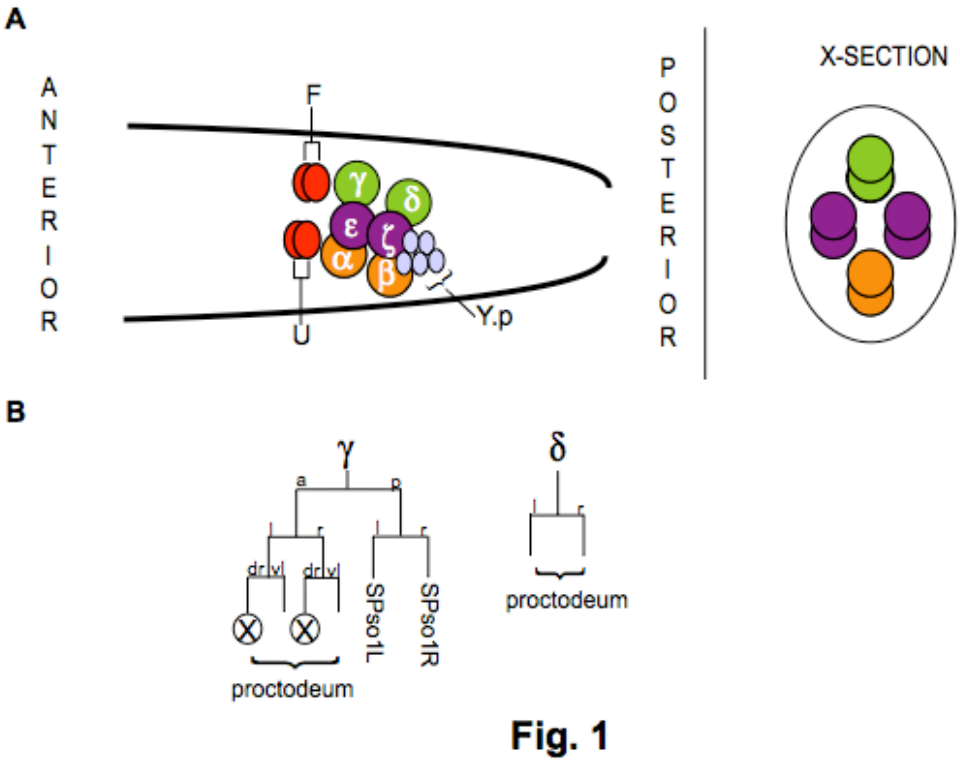

Fig. 1

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.

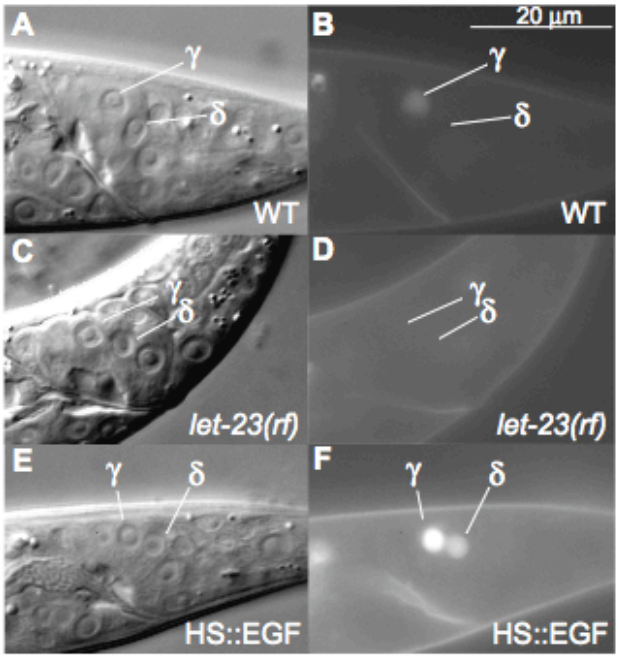
A
γ
δ
WT
B
20 µm
γ
δ
WT
C
γ
δ
let-23(rf)
D
γ
δ
let-23(rf)
E
γ
δ
HS::EGF
F
γ
δ
HS::EGF

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.

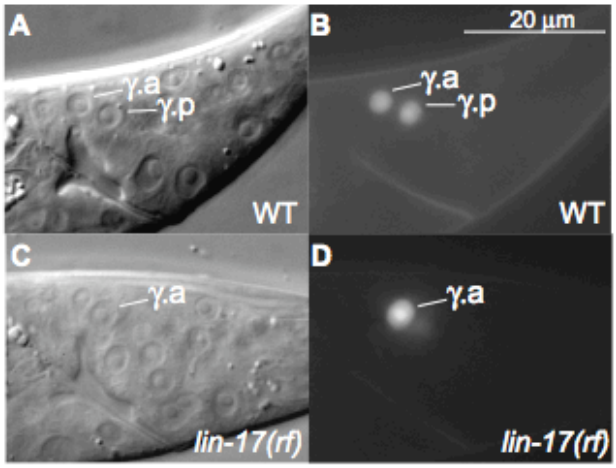

Fig. 3

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.

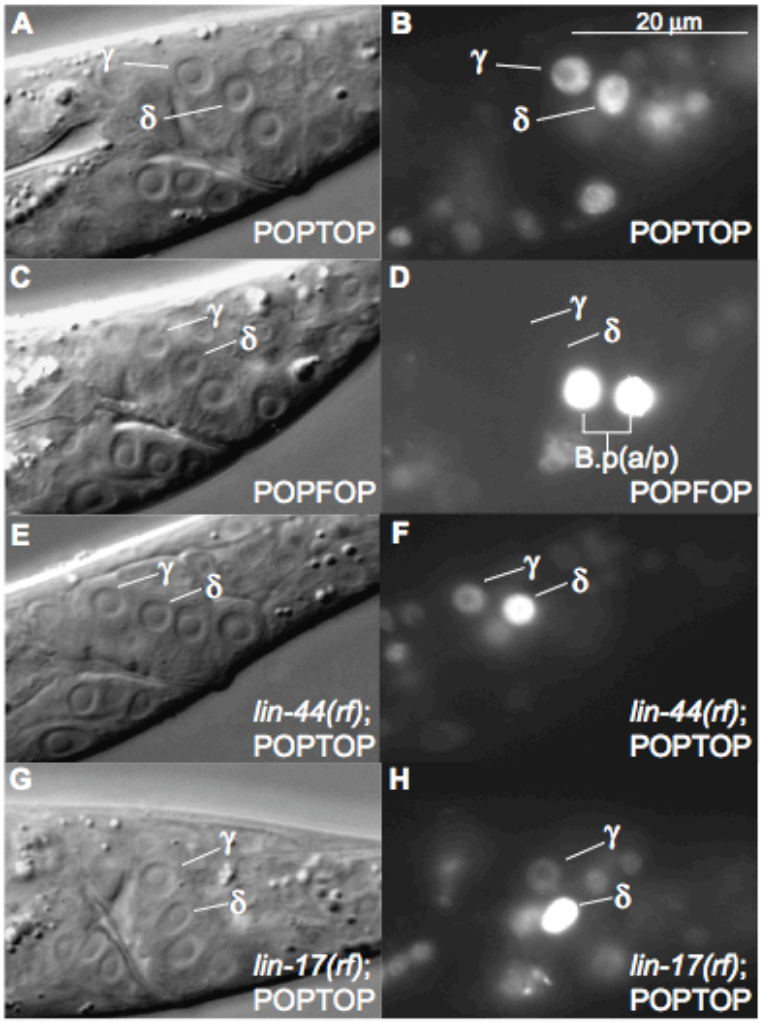
A
γ
δ
POPTOP
B
20 µm
γ
δ
POPTOP
C
γ
δ
POPFOP
D
γ
δ
B.p(a/p)
POPFOP
E
γ
δ
lin-44(rf);
POPTOP
F
γ
δ
lin-44(rf);
POPTOP
G
γ
δ
lin-17(rf);
POPTOP
H
γ
δ
lin-17(rf);
POPTOP

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.

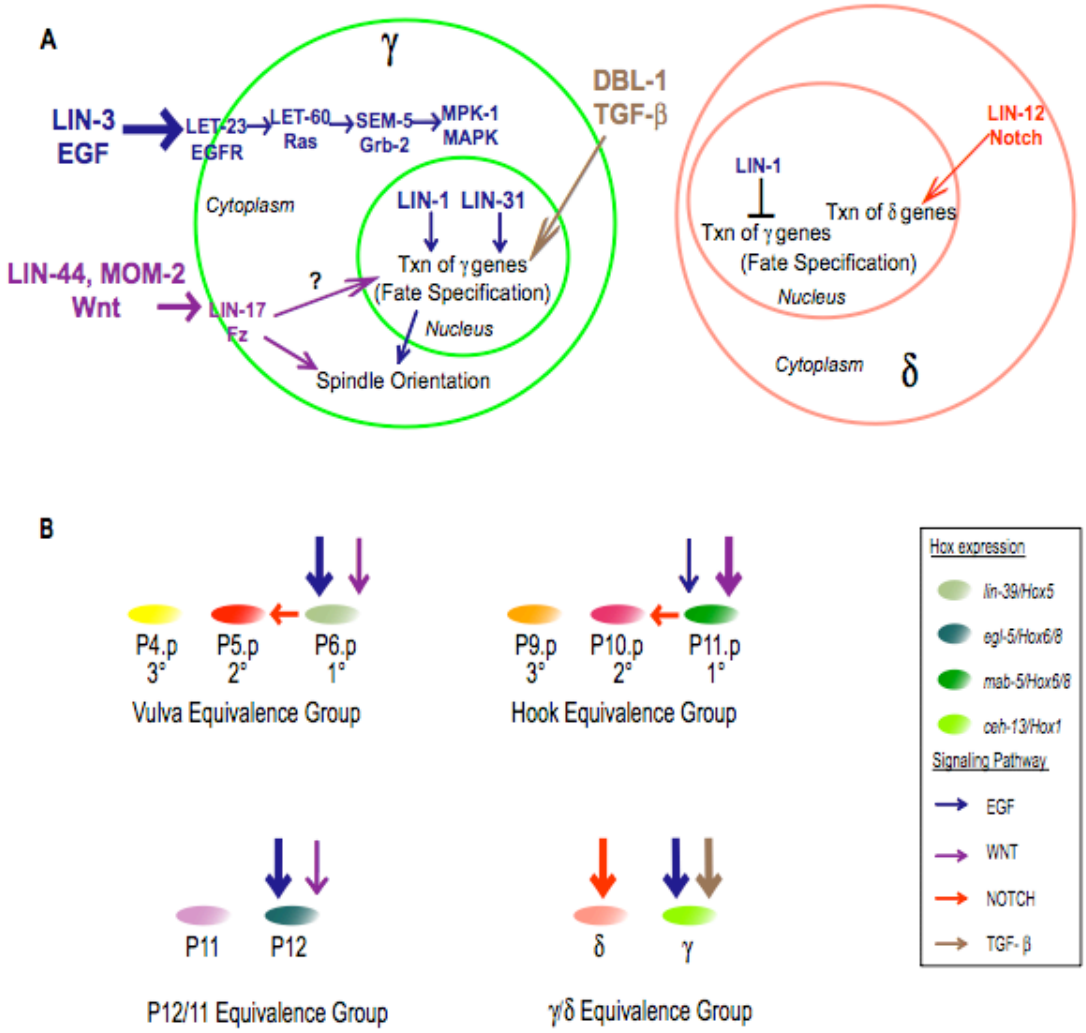
A
γ
LIN-3
EGF
LET-23
EGFR
LET-60
Ras
SEM-5
Grb-2
MPK-1
MAPK
Cytoplasm
LIN-1 LIN-31
Txn of γ genes
(Fate Specification)
Nucleus
DBL-1
TGF-β
LIN-44, MOM-2
Wnt
LIN-17
Fz
?
Spindle Orientation
LIN-12
Notch
LIN-1
Txn of δ genes
Txn of γ genes
(Fate Specification)
Nucleus
Cytoplasm
δ
B
P4.p
3°
P5.p
2°
P6.p
1°
Vulva Equivalence Group
P9.p
3°
P10.p
2°
P11.p
1°
Hook Equivalence Group
Hox expression
lin-39/Hox5
egl-5/Hox6/8
mab-5/Hox6/8
ceh-13/Hox1
Signaling Pathway
EGF
WNT
NOTCH
TGF-β
P11
P12
P12/11 Equivalence Group
δ
γ
γ/δ Equivalence Group

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.

References

Aroian, R. and Sternberg, P. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. *Genetics* **128**, 251-267.

Baird, S. E. and Ellazar, S. A. (1999). TGFbeta-like signaling and spicule development in Caenorhabditis elegans. *Dev Biol* **212**, 93-100.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev* **9**, 3149-62.

Blair, S. S. and Weisblat, D. A. (1984). Cell interactions in the developing epidermis of the leech Helobdella triserialis. *Dev Biol* **101**, 318-25.

Campos-Ortega, J. A. and Knust, E. (1990). Genetics of early neurogenesis in *Drosophila melanogaster*. *Annu Rev Genet* **24**, 387-407.

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

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

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

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992). *C. elegans* cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-4.

Eisen, J. S. (1992). The role of interactions in determining cell fate of two identified motoneurons in the embryonic zebrafish. *Neuron* **8**, 231-40.

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

Ferreira, H. B., Zhang, Y., Zhao, C. and Emmons, S. W. (1999). Patterning of *Caenorhabditis elegans* posterior structures by the Abdominal-B homolog, *egl-5*. *Dev Biol* **207**, 215-228.

Gleason, J. E., Szyleyko, E. A. and Eisenmann, D. M. (2006). Multiple redundant Wnt signaling components function in two processes during *C. elegans* vulval development. *Dev Biol* **298**, 442-57.

Green, J. L., Inoue, T. and Sternberg, P. W. (2008). Opposing Wnt pathways orient cell polarity during organogenesis. *Cell* **134**, 646-56.

Greenwald, I. S., Sternberg, P. W. and Horvitz, H. R. (1983). The *lin-12* locus specifies cell fates in *C. elegans*. *Cell* **34**, 435-444.

Herman, M. A. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. *Development* **120**, 1035-47.

Herman, R. K. (1978). Crossover suppressors and balanced recessive lethals in *Caenorhabditis elegans*. *Genetics* **88**, 49-65.

Hodgkin, J., Horvitz, H. R. and Brenner, S. (1979). Nondisjunction mutants of the nematode *C. elegans*. *Genetics* **91**, 67-94.

Howard, R. M. and Sundaram, M. V. (2002). *C. elegans* EOR-1/PLZF and EOR-2 positively regulate Ras and Wnt signaling and function redundantly with LIN-25 and the SUR-2 Mediator component. *Genes Dev* **16**, 1815-1827.

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

Hwang, B. J. and Sternberg, P. W. (2004). A cell-specific enhancer that specifies *lin-3* expression in the *C. elegans* anchor cell for vulval development. *Development* **131**, 143-151.

Immergluck, K., Lawrence, P. A. and Bienz, M. (1990). Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* **62**, 261-8.

Jiang, L. and Sternberg, P. W. (1998). Interactions of EGF, Wnt and Hom-C genes specify the P12 neuroectoblast fate in *C. elegans*. *Development* **125**, 2337-2347.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *C. elegans*. *Dev Biol* **87**, 286-300.

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

Miller, L. M., Gallegos, M. E., Morisseau, B. A. and Kim, S. K. (1993). *lin-31*, a *Caenorhabditis elegans* HNF-3/fork head transcription factor homolog, specifies three alternative cell fates in vulval development. *Genes Dev* **7**, 933-47.

Miller, L. M., Hess, H. A., Doroquez, D. B. and Andrews, N. M. (2000). Null mutations in the *lin-31* gene indicate two functions during *Caenorhabditis elegans* vulval development. *Genetics* **156**, 1595-602.

Morita, K., Chow, K. L. and Ueno, N. (1999). Regulation of body length and male tail ray pattern formation of *Caenorhabditis elegans* by a member of TGF-beta family. *Development* **126**, 1337-47.

Nilsson, L., Tiensuu, T. and Tuck, S. (2000). *Caenorhabditis elegans lin-25*: a study of its role in multiple cell fate specification events involving Ras and the identification and characterization of evolutionarily conserved domains. *Genetics* **156**, 1083-96.

Panganiban, G. E., Reuter, R., Scott, M. P. and Hoffmann, F. M. (1990). A *Drosophila* growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. *Development* **110**, 1041-50.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-16.

Rogalski, T. M., Moerman, D. G. and Baillie, D. L. (1982). Essential genes and deficiencies in the *unc-22* IV region of *Caenorhabditis elegans*. *Genetics* **102**, 725-36.

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

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

Schlesinger, A., Shelton, C. A., Maloof, J. N., Meneghini, M. and Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early *Caenorhabditis elegans* embryo without requiring gene transcription in the responding cell. *Genes Dev* **13**, 2028-38.

Singh, N. and Han, M. (1995). *sur-2*, a novel gene, functions late in the *let-60* ras-mediated signaling pathway during *Caenorhabditis elegans* vulval induction. *Genes Dev* **9**, 2251-65.

Sternberg, P. W. (2005). Vulval development. *WormBook*, 1-28.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans*. *Cell* **44**, 761-772.

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

Sternberg, P. W. and Horvitz, H. R. (1989). The combined action of two intercellular signaling pathways specifies three cell fates during vulval induction in *C. elegans*. *Cell* **58**, 679-693.

Stoyanov, C. N., Fleischmann, M., Suzuki, Y., Tapparel, N., Gautron, F., Streit, A., Wood, W. B. and Muller, F. (2003). Expression of the *C. elegans labial* orthologue *ceh-13* during male tail morphogenesis. *Dev Biol* **259**, 137-49.

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

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

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

Suzuki, Y., Yandell, M. D., Roy, P. J., Krishna, S., Savage-Dunn, C., Ross, R. M., Padgett, R. W. and Wood, W. B. (1999). A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*. *Development* **126**, 241-50.

Szuts, D., Eresh, S. and Bienz, M. (1998). Functional intertwining of Dpp and EGFR signaling during *Drosophila* endoderm induction. *Genes Dev* **12**, 2022-35.

Tan, P. B., Lackner, M. R. and Kim, S. K. (1998). MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. *Cell* **93**, 569-80.

Tiensuu, T., Larsen, M. K., Vernersson, E. and Tuck, S. (2005). *lin-1* has both positive and negative functions in specifying multiple cell fates induced by Ras/MAP kinase signaling in *C. elegans*. *Dev Biol* **286**, 338-51.

Van Buskirk, C. and Sternberg, P. W. (2007). Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat Neurosci* **10**, 1300-7.

Wagmaister, J. A., Gleason, J. E. and Eisenmann, D. M. (2006). Transcriptional upregulation of the *C. elegans* Hox gene *lin-39* during vulval cell fate specification. *Mech Dev* **123**, 135-50.

Walston, T., Tuskey, C., Edgar, L., Hawkins, N., Ellis, G., Bowerman, B., Wood, W. and Hardin, J. (2004). Multiple Wnt signaling pathways converge to orient the mitotic spindle in early *C. elegans* embryos. *Dev Cell* **7**, 831-41.

Wasylyk, B., Hagman, J. and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem Sci* **23**, 213-6.

Weisblat, D. A. and Blair, S. S. (1984). Developmental interdeterminacy in embryos of the leech *Helobdella triserialis*. *Dev Biol* **101**, 326-35.

Yu, H., Seah, A., Herman, M. A., Ferguson, E. L., Horvitz, H. R. and Sternberg, P. W. (2008). Wnt and EGF pathways act together to induce C. elegans male hook development. *Dev Biol*.