Combination of FGF and WNT Signaling Pathways Specify Positional Information During the Posterior Distal Tip Cell Migration in the *Caenorhabditis elegans* Hermaphrodites

by

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A Thesis submitted to the Faculty of Graduate Studies of

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ABSTRACT

Cell migration requires spatial and temporal regulation; this is especially critical for developmental processes such as organogenesis. Understanding the complex interactions leading to appropriate cell guidance is important since many cancers and developmental disorders stem from disruption of this fundamental process.

C. elegans is an ideal model to study and learn how cell migration is controlled during development. The distinct shape of the worm's gonad is specified by the migration of the distal tip cells (DTCs), which cap the organ. The DTCs respond to different guidance cues, such as the UNC-6/Netrin system, for its proper migration. We have previously shown that up-regulation of *unc-5* expression in the DTC is the key for the second migration phase. Also, work done in syndecan-1 mutants revealed the involvement of EGL-20/WNT and EGL-17/FGF in the proper migration of the DTC.

EGL-20/WNT is expressed by cells situated in the tail. In contrast, EGL-17/FGF is expressed by cells situated at the ventral mid-body of the worm. Both EGL-17/FGF and EGL-20/WNT act as spatial cues for the proper anteroposterior migration of specific cell types (sex myoblasts and Q neuroblasts respectively). *egl-20* and *egl-17* single mutants display very few DTC migration defects whereas. However, *egl-20; egl-17* double mutants display frequent defects in DTC migration patterns characterized by the posterior DTC failing to reflex back toward the mid-body in the third migration phase. In addition, the second, ventral-to-dorsal migration phase begins at a more anterior position than normal, suggesting that this phase is initiated precociously. We found that the more anterior position at which the posterior DTC initiates the V-D migration phase in *egl-20;*

egl-17 results not from altered timing of the initiation of the second migration phase, but from a slower migration rate during the first phase.

We propose that temporal cues like DAF-12 regulate the transitions between the longitudinal and dorsoventral migration phases, while spatial cues like EGL-17/FGF and EGL-20/WNT are critical for the proper execution of the longitudinal migration phases in the DTCs of *C. elegans* hermaphrodites.

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LIST OF ABBREVIATIONS

AKT/PKB	Protein Kinase B
Amp	Ampicillin
A-P	Antero-posterior
β	beta
bp	base pair
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
$CHCl_3$	Chloroform
C. elegans	*Caenorhabditis Elegans*
CGC	*Caenorhabditis Elegans* Genetics Centre.
cGMP	Cyclic guanosine monophosphate
cm	centimetre
^0C	degrees Centigrade
DCC	Deleted in Colorectal Cancer
ddH_20	Double distilled water
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
Dpy	Dumpy phenotype, reduced body length
DTC	Distal tip cell
D-V	Dorso-ventral
E. Coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
Egl	Egg-laying defect phenotype.
EtBr	Ethidium bromide
EtOH	Ethanol
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
F1	First generation progeny from one mating.
g	gram
GFP	Green fluorescent protein
HSN	Hermaphrodite specific neuron
HSPGs	Heparan sulphate proteoglycans
Ig	Immunoglobulin
IPTG	isopropylthiogalactoside
Kb	Kilobase
LB	Luria Bertani
L1	Larval stage one.
L2	Larval stage two.
L3	Larval stage three.
L4	Larval stage four.

LGX	Linkage group X
M	Molar
µg	microgram
mins	minutes
µl	microlitre
ml	millilitre
mM	millimolar
mm	millimetre
NaOH	Sodium hydroxide
ng	nanogram
NGM	Nematode growth medium
nM	nanomolar
N2	Wild-type *C.elegans* strain.
PCR	Polymerase chain reaction
Post.	Posterior
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
ROBO	Roundabout
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SH3	Src homology 3
SNP	Single nucleotide polymorphism
TAE	Tris-Acetic Acid EDTA buffer
Taq	*Thermus aquaticus*
TGFβ	Transforming growth factor beta
TTB	Tris Triton Buffer
Tyr	Tyrosine
Unc	uncoordinated, locomotion defect phenotype
WNT	Wingless

1. INTRODUCTION

1.1 Guidance of Migrating Cells

Cell guidance is a biological phenomenon which has a central role in developmental processes such as embryogenesis, wound healing, tumor metastasis and more recently tissue engineering. The spatial and temporal cues direct the trajectory of the migrating cell. Many different elements such as guidance cues and signaling pathways help cells to sense and respond to gradients they may encounter. Chemotaxis occurs when a cell detects a gradient of an extracellular ligand and will respond by either moving towards (chemoattraction) or away from (chemorepulsion) the gradient (Stephens, L. *et al.,* 2008).

In order for the cell to migrate, a few processes must occur to allow for the movement of the entire cell body. The cells establish a leading edge where the actin filaments polarize to create the protrusion of the pseudopodia or lamellipodia which provide traction and forward movement towards the point of interest. At the same time, myosin contraction occurs at the back of the cell resulting in retraction of the posterior end (Firtel, R.A. and Chung, C.Y., 2000). These migrating cells also secrete metalloproteinase enzymes in order to navigate through the extracellular matrix (ECM) (Shapiro, S.D., 1998).

The substrate along which growth cones and axons migrate is important not only for support but also for guidance. Neural cell adhesion molecule (N-CAM), N-cadherin and laminin are three molecules that are expressed on or around neuroepithelial and mesenchymal for proper cells migration (Dodd, J. and Jessell, T.M., 1988). N-CAM and N-cadherin are cell adhesion molecules and do not confer cell guidance due to their ubiquitous expression in the developing nervous system. However, laminin which is an

important extracellular matrix (ECM) glycoprotein has a more specific expression pattern, and has been implicated in directing cell migration (Riggott, M.J. and Moody, S.A., 1987). Laminin is also associated with Netrin/UNC-6 in the midline to establish the D-V axis (Ishii, N. 1992). It can interact with axonal glycoproteins belonging to the integrin family of receptors to provide cell adhesion (Dodd, J. and Jessell, T.M., 1988). Integrin molecules, which are surface proteins, can also mediate cell adhesion to ECM glycoproteins. Previous reports showed that blocking of integrins prevented axon extensions on laminin or ECM substrates, establishing their role in cell migration (Tomaselli, K.J. *et al.,* 1988).

There are four well known classes of molecules that function in chemotaxis; Netrin/UNC-6 (Culotti, and Merz, D.M., 1998), Slits (Rothberg, *et al.,* 1990), Semaphorins (Nakamura, *et al.,* 2000) and Ephrins (Wilkinson, 2001). These molecules provide instructive cues to migrating growth cones and pioneer axons during their migration along the anteroposteior (A-P), dorsoventral (D-V) axes or even across the midline (Killeen, M.T. and Sybinco, S.S., 2008). Along with these molecules, other secreted ligands can provide guidance as well. These include the WNT family, Sonic headgehog (Shh), TGF-β as well as FGFs (Killeen, M.T. and Sybinco, S.S., 2008). The WNT and FGF signaling pathways help establish an A-P axis for axonal migrations (Silhankova, M. and Korswagen, H., 2007; Goldfarb, M., 1996). Together these signaling pathways have also been found to regulate the expression of Hox genes, which provide more regional specificities, as well as Shh expression during limb development (McGinnis, W. and Krumlauf, R., 1992; Goldfarb, M., 1996). Molecules such as PI3K (phosphatidylinositol-3-kinase) have been reported to be heavily involved in signal

transduction during cell migration of macrophages, neutrophils and mammalian cells (Firtel, R.A. and Chung, C.Y., 2000; Stephens, L. *et al.,* 2008). The kinase activity of PI3K is essential for mediating creating rapid intracellular responses and activating other downstream elements. Elements such as Akt/PKB and PTEN are also important in signaling so that the appropriate rearrangements of the actin and myosin filaments of the cytoskeleton can mediate cell migration (Firtel, R.A. and Chung, C.Y., 2000; Stephens, L. *et al.,* 2008). G protein coupled receptors have recently been shown to be responsible for mediating cell migration to chemoattractants and cAMP in *Dictyosteium* amoebae, neutrophils macrophages and fibroblasts (Firtel, R.A. and Chung, C.Y., 2000).

The migrating neuron or growth cone is constantly presented with multiple guidance cues to which it responds to regulate its migration path. Receptors at the surface of the cell are responsible for the response of the cell during cell migration. These receptors, along with integrin adhesion receptors and integrin cytoskeleton linkage, can redistribute themselves to better serve the cell in response to chemotaxis (Lauffenburger, D.A., 1996). The response of a receptor to a certain extracellular cue can vary depending on numerous factors such as distance from the chemotactic molecule and intracellular levels of cyclic nucleotides to name a few. For example, the activity of Netrin/UNC-6 system has been shown to be regulated by phosphorylation of its receptors UNC-5 and UNC-40/DCC. Although UNC-5 is dependent on Netrin/UNC-6 to mediate repulsion, its level of phosphorylation affects the degree to which it can respond to the source of Netrin/ UNC-6 (Tong, J. *et al.,* 2001).

1.2 *Caenorhabditis elegans*

The nematode *C. elegans* was first introduced as an animal model by Sydney Brenner in 1965 (Brenner, S., 1974). It is a free-living non-parasitic organism found in the soil in all parts of the world. Of all the metazoans, *C. elegans* is one of the most completely understood organisms regarding its genetics, anatomy, development and behavior (Wood, W.B., 1988). It was also the first multicellular organism to have its entire genome sequenced and published (Hodgkin, J., *et. al.,* 1998). The organism has a short life cycle of approximately three days and has very large brood sizes, which make it an ideal model to handle in the lab. There are two sexes in *C. elegans*: hermaphrodites and males. The hermaphrodites are capable of self-fertilization since they produce both oocyte and sperm and do not need the males in order to make progeny. Males of *C. elegans* occur spontaneously at a frequency of about 1 in 500 progeny and can mate with the hermaphrodites. The number of males can be maintained by crossing them to hermaphrodites. On their own, hermaphrodites can produce up to 300 eggs through self-fertilization whereas the brood size is much larger if the hermaphrodites mate with a male and can produce over 1000 eggs. The mating of hermaphrodites and males is essential when crossing different strains. *C. elegans* has a very simple anatomy (Wood, W.B., 1988). Both the hermaphrodites and males have a predetermined somatic cell body count of 959 and 1031, respectively. These somatic cells differentiate into all the different organs and tissues necessary to make the worm such as hypodermis, muscle, intestine, reproductive, excretory and nervous systems. In fact, the nervous system is composed of 302 of these somatic cells and detailed maps of the wiring of these neurons exist for *C. elegans* (White, J.G. *et al.*, 1986).

Embryogenesis occurs in two steps for *C. elegans*. First the embryo goes through cell proliferation and organogenesis before going through morphogenesis. Early embryogenesis (first 7 hours) consists of cells dividing, cellular movement and some cell death in a very precise temporal and spatial pattern. This pattern is invariant from one embryo to the next and gives rise to a specific number of cells with predetermined fates (Wood, W.B., 1988). The *C. elegans* organism precisely predetermines the fate of every cell. This is also true for cells that are destined for cell death. Research has been able to determine which cells are destined for cell death and has led to the opportunity to study programmed cell death (PCD) *in vivo*. In fact Syndney Brenner and his colleagues were awarded the 2002 Nobel Prize in Physiology or Medicine for determining the genetic regulation of organ development and PCD (Putcha, G.V. and Johnson, E.M., 2004).

Cell proliferation slows down approximately half way through embryogenesis to give way to body elongation, neuronal growth and interconnection as well as cuticle formation. Eggs are laid three hours after fertilization. However, embryogenesis goes on for a total of 14 hours before the worm can hatch and go though larval development, which takes another 50 hours. Once the larvae have hatched they enter into the first larval phase (L1) of post-embryonic development. At this phase, worms are only about 250μm long and hermaphrodites contain 558 cells and males contain 560 cells in total. The larva go through three stages which are referred to as larval stages L1, L2, L3 and L4 before becoming mature adult worms (Wood, W.B., 1988).

C. elegans has six haploid chromosomes referred to (for historical reasons) as linkage groups (Brenner, S., 1974). Hermaphrodites are diploid for all of the five autosomal chromosomes (5 A/A) as well as the sex chromosome (X/X). Although males are diploid

for the five autosomal chromosomes (5 A/A) as well, they have one less chromosome than the hermaphrodites. Instead of having two sex chromosomes (X/X) they only have one (X/O). As mentioned previously, males are a spontaneous occurrence in the hermaphrodite population and arise through X chromosome non-disjunction during meiosis (Wood, W.B., 1988).

The adult hermaphrodite and male worms are approximately 1mm in length and have an entirely transparent body throughout development. These characteristics allow the researcher to readily observe general phenotypes under a dissecting microscope and more refined cellular details using light microscopy with differential interference contrast (DIC) optics.

As mentioned before, *C. elegans* is easily maintained in the lab. It grows on agar plates and is sustained by feeding with *E. coli*. For larger scale experiments the worms can be grown in liquid culture. In optimal conditions, abundant food and at a temperature of 20°C, the worms have a lifespan of about 17 days once they have reached adulthood. However, under unfavorable conditions, lack of food and overcrowding, the worms can undergo an alternative developmental pathway called the dauer stage (Wood, W.B., 1988). In this process, worms stop eating and developing in early larval stage (L3) and can survive up to three months without further development. The worm can resume development when it senses that conditions have become favorable and that food is readily available (Wood, W.B., 1988).

C. elegans is a very valuable animal model. So far it has been used in a wide variety of different research fields to study processes such as apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism and ageing (Kaletta, T. and Hengartner, M.O.,

2006). More recently, *C. elegans* has also been used to model certain human disorders such as diabetes, depression and Alzheimer's disease to get a better understanding of the molecular implications and *in vivo* interactions involved (Sundaram, M. and Greenwald, I., 1993; Nakae, J. *et al.*, 2002; Ranganathan, R. *et al.*, 2001). The conservation of key genes and proteins of important signaling pathways coupled with the simplicity of the organism and the knowledge at hand make it an ideal model for basic research.

1.3 Gonad Development

C. elegans gonad development occurs in three sequential phases. The alternation between migrations along the antero-posterior and dorso-ventral axes of the worm gives rise to mirror-image C shapes in both the anterior and posterior gonad arms. The gonad arms start their migration from the ventral mid-body of the animal during the late L3 stage. Both the anterior and posterior gonad arms are capped by a single leading cell referred to as the distal tip (DTC). The DTCs extend out along the ventral bands of body wall muscle (BWM) of the animal (the first migration phase) about 25 hours after hatching (Wood, W.B., 1988). At approximately 30 hours (L3/L4 stage transition) after hatching, the DTC enters the second migration phase where it takes a 90° turn to migrate from the ventral to the dorsal band of BWM of the animal. Once the dorsal side has been reached, at 33-34 hours after hatching, the DTC makes a second 90° turn to migrate back to the mid-body along the dorsal side of the worm (third phase)(Figure 1-A)(Su, M. *et al.*, 2000). Mutations of *unc-5*, *unc-6* or *unc-40* prevent the DTC from completing its second ventral-to-dorsal migration phase (Hedgecock, E.M. *et al.*, 1990; Culotti and Merz, D.M., 1998). In these mutants, the DTC fails to turn to the dorsal side of the

animal and completes the third migration phase along the ventral side of the animal (Figure 1-B). This migration defect is easily observed due to the fact that the misshapen gonad arm forces the intestine of the worm dorsally. Under a dissecting microscope one can see a clear patch along the ventral side of the animal and large numbers of animals can be quickly analyzed for phenotypes resulting from defects in the DTC migration.

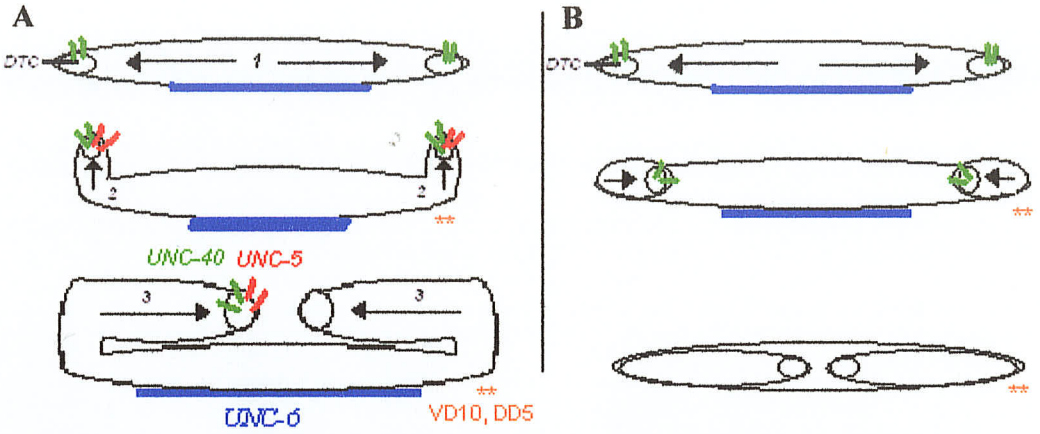


Figure 1. Representation of the gonad arm migration phases in *C. elegans* hermaphrodite.
A-represents a wild type strain. The DTCs lead the gonad arms through all 3 migration phases (from top, being the first phase, to bottom) starting from the ventral mid-body, outward. In the second migration phase, the DTCs turns at a 90° angle over the VD10 and DD5 neurons and migrates from ventral to dorsal side. This ventral to dorsal migration is driven by the expression of UNC-5 and UNC-40 in the DTCs, which mediate the repulsion of the DTCs away from the UNC-6 ventral expression. Finally, the third migration phase takes place along the dorsal side back to the mid-body of the animal. The mature adult gonad forms a characteristic mirror image C-shape. **B**-represents the characteristic migration defect caused by mutations of *unc-5*, *unc-6* and/or *unc-40* mutants. The DTC fails to migrate from ventral to dorsal during the second migration phase and forces the gonad arms back along the ventral side during the third migration phase creating a clear patch in the ventral side of the worm.

1.4 Netrin Guidance System

UNC-6/Netrin was first described in *C. elegans* (Ishii, N. *et al.,* 1992). The Netrin guidance system is a highly conserved group of molecules which are essential for both short and long-range cell guidance (Culotti J.G and Merz D.C., 1998). Vertebrates and invertebrates express netrins at the ventral midline and they are involved in the early migration of axons (Wadsworth W.G., 2002). Netrins can act both as attractant and repellent cues. Netrin 1 and Netrin 2 in chick brain have been shown to act as attractants and repellents in different cells. The DCC/UNC-40 receptor attracts and UNC-5 receptor repels the cells from the UNC-6 source. The most notable function of netrins is to outline the midline for growing axons in the invertebrate and vertebrate system. (Livesey, F.J., 1999).

1.4.1 UNC-6

UNC-6 is the *C. elegans* homologue of the mammalian netrin proteins. The *unc-6* gene encodes a 591 amino acid protein with the N terminal (4 of the 5 structural domains) end being homologous to laminin protein and a unique C terminal end (fifth domain) (Wadsworth W.G., 1996; Ishii N., 1992). UNC-6 co-polymerizes with laminin subunits in the basement membrane to form a stable ventral-dorsal gradient where it provides an instructive cue for migrating cells and axons (Ishii, N. 1992). Ventral epidermal and neuronal cells secrete UNC-6 to provide directional information concerning the midline of the organism (Hedgecock, E.M. *et al.*, 1990). Genetic mutants of UNC-6/Netrin ligand or receptors create partially penetrant phenotypes which suggest that other guidance cues are involved in cell migration along with Netrin guidance system. These mutants display ventro-dorsal migration defects but not longitudinal migration defects

(Ishii, N. *et al.,* 1992). This suggests that the gradient traditionally known to guide circumferential migrations also helps to specify ventro-dorsal positioning of longitudinal migrating nerves (Xing-Cong, 1998). In *C. elegans*, PVT and AVG neurons (at either end of the worm) express *unc-6* along with ventral neurons. *Unc-6* expression by epidermal cells is sufficient for ventro-dorsal migration but PVT expression of *unc-6* is required for positional information (special cue) (Xing-Cong, 1998). The phylogenetic conservation of ventral expression of netrin (specific spatial source of signal) in developing CNS is important. Experiments using widespread expression of netrin create many defects indicating that the precise localization of netrin is very important (Xing-Cong, R. *et al.,* 1998).

1.4.2 UNC-5

C.elegans is not the only organism that depends on the Netrin guidance system for proper cell migration. Mutations of one mammalian homologue of UNC-5 gene (UNC5H3), which is located at the murine rostral cerebellar malformation (rcm) locus, also affects the cell migration of granule cells in the cerebellum thereby causing developmental defects (Ackerman, S.L. *et al.* 1997).

The response of the migrating cells to the ventral source of UNC-6/Netrin is dependent upon the receptors that the cell expresses at its surface. One of the receptors mediating a response to the UNC-6/Netrin ligand is UNC-5 which causes repulsion away from the UNC-6/Netrin source (Ackerman, S.L. *et al.,* 1997). UNC-5 shares many common structural features with the three mammalian subtypes RCM, UNC5H1 and UNC5H2 (Tong, J. 2001). This 919 amino acid transmembrane protein has an extracellular domain containing two immunoglobulin domains along with two

thrombospodin type 1 domains. The single transmembrane domain is followed by the cytoplasmic domain consisting of a juxtamembrane domain and a conserved ZU-5 domain (Tong, J. 2001). The extracellular component of the protein suggests its involvement as a cell adhesion molecule or as a receptor (Livesey F.J., 1999).

In *C. elegans unc-5* is expressed in all 5 classes of motoneurons that project axons dorsally (DA, DB, DD, VD and AS) along with the DTCs and sensory neurons in the head. In DTCs, *unc-5* expression is not observed during the first migration phase and is only up-regulated during the second migration phase (Su, M. *et al.*, 2000). However, ectopic *unc-5* expression in the first migration phase driven by the *emb-9* promoter causes the DTC to aberrantly initiate the second migration phase at a more anterior position than the wild-type DTC (Su, M. *et al.,* 2000). Therefore, expression of *unc-5* is necessary and sufficient for the second migration phase of the DTCs.

UNC-5 has been shown to be the minimum requirement to direct migrating cells from ventral to dorsal, away form the UNC-6 source (Keleman, K. and Dickson, J.B., 2001). Ectopic mis-expression of *unc-5* in cells that do not usually migrate dorsally will cause migration in this direction dependent on the source of UNC-6/Netrin (Hamelin, M. *et.al.,* 1993). In an *unc-6* mutant background, these cells expressing ectopic *unc-5* migrate normally, indicating that the UNC-5 receptor requires *unc-6* expression for proper ventro-dorsal migration. This finding also suggests that growth cones that usually migrate in different directions selectively express different extracellular receptors depending on multitude of cues being expressed surrounding them, but share similar intracellular downstream signaling (Tong, J. 2001).

Phosphorylation is another mechanism of regulation for *unc-5* and *unc-40/DCC* expression since both UNC-5 and UNC-40/DCC are phosphorylated on tyrosine residues (Tong, J. *et al.,* 2001). The phosphorylation level of both these receptors is thought to help regulate their ability to respond to their ligand UNC-6/Netrin. It is also reported that UNC-5 apparently has multiple potential tyrosine phosphorylation sites and that there might be other ligands that can interact with UNC-5 and in turn enable it to respond to UNC-6/Netrin chemorepulsion (Tong, J. *et al.,* 2001).

1.4.3 UNC-40

UNC-40/DCC (deleted in colorectal cancer) is the second receptor for UNC-6/Netrin ligand. This single transmembrane protein is homologous to neogenin and contains four immunoglobulin domains along with six type III fibronectin (FN) repeats and a cytoplasmic tail (Keino-Masu, K. *et al.*, 1996). Unlike UNC-5, UNC-40/DCC mediates the attraction of cells towards the netrin source in a cell-nonautonomous manner (Su *et al.*, 2000). However, when co-expressed with UNC-5 it has also been shown to mediate repulsion. It is thought that UNC-5 can convert UNC-40 to a repellant receptor, overriding the attraction role of UNC-40/DCC (Tong, J. 2001). Although UNC-40 is largely dependent on the UNC-6 ligand to mediate cell guidance it has been shown that it is also regulated by other interactions. Heparan sulfate and heparin molecules seem to be important for complete signaling through DCC in mammals (Livesey F.J., 1998). In humans, UNC-40/DCC is found on chromosome 18q and is found to be commonly deleted in metastatic colorectal cancer. It is highly expressed in the developing CNS but is also normally expressed in the gastrointestinal tract (Livesey F.J., 1998).

1.5 DAF-12

Research done on the Netrin guidance system and the involvement of *daf-12* gene in cell guidance suggests that *daf-12* is involved in the up-regulation of *unc-5* transcription at the end of the first migration phase enabling the dorsal migration of the DTC can migrate to the dorsal side (Su, M. *et al.*, 2000). Furthermore, it is thought that *daf-12* can disrupt the DTC by blocking the progression of the larval stage-specific developmental programs which delay or block the L3 and L4 stages (Su, M. *et al.*, 2000). These stages correspond to the late first migration phase and into the second migration phase, when the DTC starts to turn towards the dorsal side (Figure 1-A). This causes the DTC to fail to initiate the second or third phase of migration because of the absence of or delay of the progression into the L4 developmental program (Su, M. *et al.*, 2000). Thus, DAF-12 is critical not only in the proper progression of the different developmental stages of the developing worm but also for the expression of *unc-5* at the appropriate time for the second migration phase of the DTCs.

Mutations of *daf-12* also render the worms unable to enter the dauer stage when environmental conditions are unfavorable and also display a certain percentage of DTC migration defects (Antebi, A. *et al.*, 2000). Heterochronic genes such as *lin-4*, *lin-14*, *lin-28*, *lin-29* and *daf-12* are responsible for the timing of many different post-embryonic developmental events. Mutations of any of these genes can cause precocious or retarded onset of certain key developmental events such as neuronal migration, vulva development, dauer larva formation and terminal differentiation of the hypodermis (Ambros, V. 1997; Rougvie, A., 2001). Some of these genes are linked to each other through genetic epistasis which means that these genes have an effect on each others

expression dependent on if each of their respective developmental stage has been properly followed through (Ambros, V. 1997; Rougvie, A., 2001).

The *daf-12* gene encodes a nuclear hormone receptor that regulates larval development. More precisely it is involved in larval stage-specific development programs of many different somatic cell types and is essential for the onset of the dauer pathway, developmental age and longevity of the adult worm (Ambros, V., 1997). DAF-12 has three isoforms, two (12A1 and 12A3) of which have DNA binding domains (DBD) and a ligand binding domain (LBD). The third isoform only has a LBD (Antebi, A. *et al.*, 2000).

The *daf-12* expression pattern throughout *C. elegans* development and body was described by Antebi *et al.* using a GFP fusion construct in frame with exon 1 of this gene (Antebi, A. *et al.*, 2000). The expression of *daf-12* is widespread in many tissues such as the epidermis, vulva, somatic gonad, intestine, pharynx and sex myoblasts. The expression persists from early embryonic stage to adulthood with a peak at the L2 developmental stage. The expression pattern of *daf-12* in the somatic gonad was also characterized. Similar to what was observed in the rest of the worm, *daf-12* expression starts in the early embryonic stage more specifically in early L1 in the Z1 and Z4 gonad precursor cells (Antebi, A. *et al.*, 2000). Their descendents, the somatic gonadoblasts, also express *daf-12* in the L2 stage. The expression of *daf-12GFP* persists into the gonadoblasts descendents and in the DTCs during the L3 stage and into early L4. In the adult hermaphrodite, *daf-12GFP* is most noticeable in the uterus (Antebi, A. *et al.*, 2000). Thus, DAF-12 is expressed in the DTCs at the time of turning during the second migration phase, consistent with the genetics.

1.6 Regulation of DTC Migration Pattern

The DTCs follow a very organized and regulated signaling pathway in order to achieve the mirror-image c shape of the adult hermaphrodite gonad arms. Identifying genes that disrupt this pattern will help to elucidate these tightly regulated signaling cascades that lead to DTC migration. Work done by Merz D.M, *et al.* (2003) has previously shown that UNC-52, a homologue of heparan sulfate proteoglycan (HSPG) perlecan, is involved in directing the DTC during its migration phases (Merz, D.M. *et al.* 2003). Previous reports had linked UNC-52 with the proper formation and maintenance of basement membrane and distribution of secreted growth factors (Arikawa-Hirosawa, E. *et al.,* 1999). The secreted growth factor-like molecules tested were UNC-129/TGFβ, DBL-1/EGF, EGL-17/FGF and EGL-20/WNT, which all belong to classes of growth factors previously linked to regulation through HSPG (Baeg, G.H. and Perrimon, N., 2000; Merz, D.M. *et al.* 2003). Merz, *et al.* were able to demonstrate that mutations of *unc-52* causes DTC migration defects through mis-regulation of growth factor signaling pathways.

More recent work implicating another HSPG, SDN-1/Syndecan in *C. elegans*, showed that mutant *sdn-1*affects DTC migration and is dependent on two secreted growth-factor like molecules: EGL-17/FGF and EGL-20/WNT (Schwabiuk, M.L., 2006). Together these reports have helped identify secreted molecules (EGL-17/FGF and EGL-20/WNT) that are involved in the proper migration of the DTC. Understanding how all the pieces of the puzzle fit together to complete the numerous genetic and molecular interactions that lead to cell migration is still a very active area of *C. elegans* research.

1.7 FGF/EGL-17

EGL-17/FGF is involved in many cell migration events in *C. elegans* development. The Sex Myoblasts (SMs) migrate along the A-P axis of the worms during post embryonic development and are dependent on 2 mechanisms for migration; gonad dependent guidance and gonad independent guidance. The gonad independent guidance mechanism is more broad range whereas the gonad dependent guidance mechanism is more precise (DeVore D.L. *et al.*, 1995). EGL-17/FGF is part of the gonad dependent mechanism and it is expressed in the developing gonad as well as in the vulva and acts as a chemoattractant to guide the SMs during their migration (Te-Wen Lo, *et al.*, 2008). The SMs migrate anteriorly from their birthplace and end their migration over the centre of the gonad structure where they complete their differentiation and division (Burdine, RD. *et al.*, 1997). EGL-15/FGFR mediates the SM migration and differentiation since without it the SMs are unable to differentiate. However, activation of the downstream PI3K signaling pathway can help partially suppress the effect of *egl-15* mutations (Huang, P. and Stern, M., 2005). Mutations in *egl-15* or *egl-17* disrupt the proper distribution of SMs along the A-P axis and SMs have a posterior displacement compared to the wild-type (DeVore, D.L. *et al.*, 1995). More recently, it has been shown that specific mutations of *egl-17* and *egl-15* do not just halt SM migration but actually repel them. This suggests that EGL-17/FGF not only has a chemoattractant role but also has a repulsion mechanism that can make the SM migrate anteriorly to assume their proper position (Te-Wen Lo *et al.*, 2008). Another possibility regarding the switch between attraction/repulsion of EGL-17/FGF protein was put forward by Fleming and his group. Different components of signaling transduction can have an impact on attraction or

repulsion action of EGL-17/FGF for example: phospholipase Cγ (PLCγ) and phosphoinositide 3-kinase (PI3K) can potentially function downstream of EGL-15/FGFR (Fleming T.C. *et al.*, 2005). The co-activation of both these components is required for chemoattraction of spinal neuron *Xenopus* (frog) expressing exogenous rat TrkA (receptor tyrosine kinase) (Ming, G. *et al.,* 1999). This or a similar mechanism could also be the mode of action that regulates the attractant/repellant function of EGL-17/FGF (Fleming T.C. *et al.*, 2005).

To date, as many as 22 different mammalian Fibroblast Growth Factors (FGFs) ligands have been described in a variety of vertebrates and invertebrates (Coumoul X. and Deng C., 2003). They are involved in a variety of developmental processes such as proliferation of the mesoderm, ectoderm and endoderm as well as cell motility, differentiation and extension of neurites (Mason I J., 1994). The FGF ligands bind to their respective FGFRs (receptors), which are transmembrane protein tyrosine kinase receptors with an extracellular immunoglobulin-like domain for FGF recognition (Goldfarb M., 1996). The FGFRs dimerize upon ligand binding, and autophosphorylate key tyrosine residues. Adaptor proteins such as SEM-5 (Grb2) bind to the phosphorylated receptors and mediate signaling to LET-60/Ras. This in turn activates the ERK (extracellular signal-regulated kinase)/MAPK (mitogen-activated protein kinase) signaling pathways (Kokel, M. *et al.*, 1998). This cascade of RTK-Ras-MAPK signaling is evolutionary conserved and is critical in contributing to cellular events (Goldfarb M., 2001) (Figure 2). The FGFs are key players in vertebrate embryonic growth and are part of a variety of developmental processes like growth and shaping of tissues and organs, limb development and bone growth (Goldfarb, M., 1996). They are also involved in

establishing the A-P axis in the developing CNS of embryos (Goldfarb, M., 1996). For optimal signaling through the FGFRs, previous studies have show that FGFs require the presence of heparin or heparan sulfate proteoglycans (HSPG). However not all HSPGs are beneficial for FGF/FGFR binding. Proteins such as syndecan and glypican, which are HSPGs, decrease FGF receptor binding (Mason, I. J., 1994). Human FGFRs are central players in early development. They have also been shown to be involved in cancer development. (DeVore, D.L. *et al.*, 1995). In *C. elegans* there is only one FGFR (EGL-15) and two FGF ligands (EGL-17/FGF and LET-756) that have many different roles in fluid balance retention, guidance of migrating SMs, guidance of CAN neurons by repulsion, as well as roles in axon outgrowth and maintenance of axon bundles (Fleming, T.C. *et al.*, 2005). EGL-15 (EGg-Laying defective). The only FGFR in *C. elegans* (EGL-15), is very similar to human FGFR 1 and 2 (DeVore, D.L. *et al.*, 1995).

CLR-1 (CLeaR) is a receptor tyrosine phosphatase which negatively regulates EGL-15/FGFR (Kokel, M. *et al.*, 1998). Mutations of *clr-1* are lethal due to increased signaling through EGL-15/FGFR (DeVore, D.L. *et al.*, 1995). Clr worms are recognizable by their fluid filled and clear bodies (where their name comes from) as compared to the denser and dark bodies of wild-type animals. The two FGFs in *C. elegans*, EGL-17/FGF and LET-756/FGF, act through two different isoforms of EGL-15/FGFR resulting from alternative splicing: *egl-15(5A)* and *egl-15(5B)* respectively. Although both FGFs have their respective receptor isoforms, EGL-17/FGF and LET-756/FGF can compensate for each other by binding to the other receptor (Fleming, T.C. *et al.*, 2005). LET-756 is part of the estimated 25% of genes that are essential for *C. elegans*. Mutations of this gene result in a lethal phenotype whereas mutations of *egl-17*

only result in SM migration defects (Roubin, R. *et al.*, 1999). EGL-17/FGF is also involved in the migration of CAN neurons along the A-P axis of the worm. CAN neurons are born in the head and migrate to the posterior of *C. elegans*. EGL-17/FGF acts as a repellent for CAN neuronal migration along the A-P axis and is an instructive cue for the CAN migration since global expression of the protein increases CAN migration defects. EGL-15/FGFR acts cell autonomously for proper CAN migration. (Fleming, T.C. *et al.*, 2005).

DPY-22 has been reported to be a negative regulator of the RTK/Ras signaling pathway. However, so far it has only been shown to negatively regulate LET-23/EGFR which is another tyrosine kinase receptor similar to EGL-15 (Aroian, R.V. *et al.*, 1990). LIN-15 is another negative regulator of LET-23/EGFR. It is involved in the proper development of the single vulva situated at the ventral mid-body of *C. elegans* hermaphrodites. In null mutations of *lin-15* the 6 vulvul precursor cells all assume vulval fates creating a multivulva (Muv) phenotype (Huang, L.S. *et al.*, 1994). There are two transcripts of *lin-15*, A and B, however it is thought that they have overlapping roles in vulval fate induction. Both transcripts A and B need to be mutated to obtain the Muv phenotype (Clark, S.G. *et al.*, 1994). LIN-15 does not act cell-autonomously and is secreted by the surrounding hypoerdermis of the anchor cell (Herman, R.K., and Hedgecock, E.M., 1990).

SEM-5 is also a downstream effector of the tyrosine kinase receptor signaling pathway (Downward, J., 1994). It has been shown to interact with both the LET-23/EGFR and EGL-15/FGFR activated receptors (Schutzman, J.L., *et al.*, 2001). The SEM-5 protein is homologous to the mammalian Grb2 (growth factor receptor-bound

protein 2) and is an adaptor protein made up of a Src-homology 2 domain (SH2) flanked by two SH3 domains (Worby, C. and Margolis, B., 2000). Once the tyrosine kinase receptors (LET-23/EGFR and EGL-15/FGFR) have been activated through ligand binding (LIN-3/EGF and EGL-17/FGF or LET-756/FGF, respectively), the SEM-5 adaptor protein associates with the autophosphorylated receptors. It then mediates signaling to the downstream LET-60/Ras protein which activates the MAPK (mitogen-activated protein kinase) signaling pathway (Worby, C. and Margolis, B., 2000). This signaling cascade has been previously shown to be involved in processes such as vulva induction, proliferation, differentiation as well as SM and axon migration (Sundaram, M.V., 2006). It has also been determined that SEM-5 can act as a positive or negative regulator of the downstream Ras signaling depending on the interactions with various other signaling molecules (Worby, C. and Margolis, B., 2000).

PTP-2 (Shp-2) cytosolic tyrosine phosphatase is a positive regulator of receptor tyrosine kinase induced signaling. It is found to be both essential for oogenesis and as a supporting signaling component of vulval development in *C. elegans* (Gutch, M.J. *et al.*, 1998). It is thought to associate with a putative phosphotyrosine-containing motif of the RTK. Like SEM-5, PTP-2 contains an Src homology 2 domain (SH2) containing tyrosine phosphatases (SHP). In mice, disruption of the SH2 domain of SHP2 protein creates severe defects during gastrulation. This is thought to be the result of a decrease in the of MAPK activation through FGFR in these mice (Gutch, M.J., 1998).

The MPK-1 protein works downstream of LET-60/Ras in the RTK signaling pathway of vertebrates and invertebrates (Pelech, S.L. and Sanghera, J.S., 1992). This MAPK is also known as the extracellular signal-regulated kinases (ERKs) and is known to

phosphorylate many different proteins, when activated (Lackner, M.R *et al.,* 1994). In the vertebrate model, MAPK translocates to the nucleus and phosphorylates transcription factors such as Elk-1 and NF-IL16 which in turn activate the transcription of two immediate early genes: *c-fos* and *Il-6.* Blocking of the MAPK protein inhibits fibroblast proliferation which indicates the importance of this protein in the RTK signaling pathway (Marais, R.J. *et al.,* 1993; Nakajima, T. *et al.,* 1993). In *C. elegans* there are two known MAPK genes, *mpk-1* and *mpk-2.* These MPK proteins also have been found to translocate to the nucleus and activate transcription of genes essential for controlling vulval induction (Lackner, M.R *et al.,* 1994).

Another potential downstream component of the RTK/Ras signaling pathway is the novel nuclear protein LIN-25. This gene is required for the VPC (vulval precursor cells) fate specification and acts downstream of LET-60/Ras as a nuclear protein (Tuck, S. and Greenwald, I., 1995). LIN-25 works with another novel nuclear protein, SUR-2 as a mediator complex to link certain sequence specific DNA binding proteins to the RNA polymerase II complex, it could also act as a transcription factor or recruit the appropriate factors for transcription (Boyer, T.G. *et al.,* 1999; Tuck, S. and Greenwald, I., 1995). LIN-25 may be a target of MPK-1 (Tuck, S. and Greenwald, I., 1995).

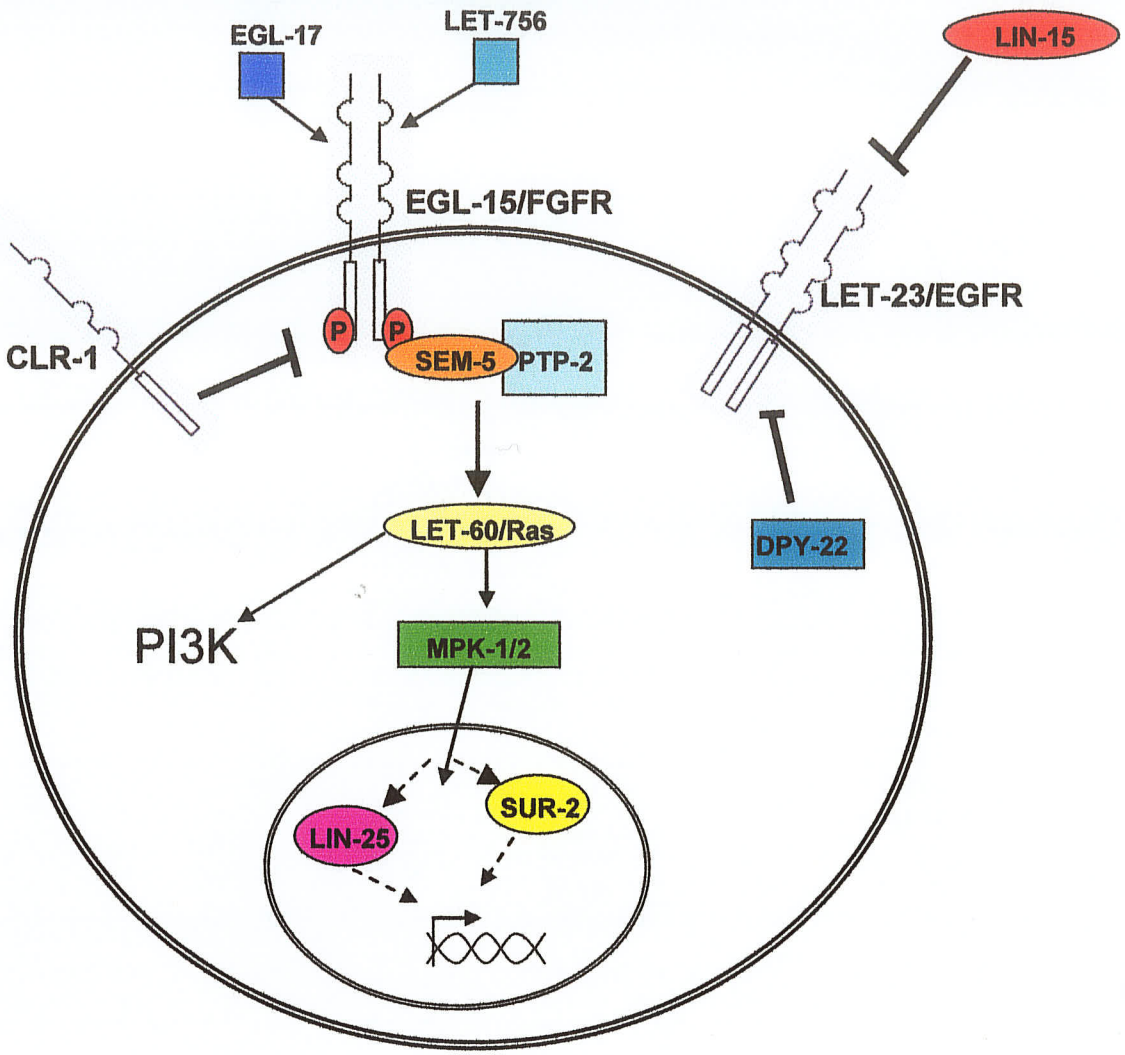


Figure 2. RTK/Ras/MAPK signaling pathway.
This figure is a cartoon representation of the FGF signaling pathway. See text for references.

1.8 WNT/EGL-20

The WNT signaling pathway is highly conserved in vertebrates and invertebrates. It is essential for processes such as cell proliferation, migration, polarity, differentiation and axon outgrowth (Eisenmann, D.M., 2005). In vertebrates and invertebrates, these conserved secreted glycoproteins act as graded morphogens and guide axons along the A-P axis. They can control a wide variety of developmental processes through the 'canonical' and 'non-canonical' pathways (Silhankova, M. and Korswagen, H., 2007). Research shows that certain colon cancers and oncogenesis in other tissues stem from over-activation of the WNT signaling pathway (Eisenmann, D.M., 2005).

EGL-20/WNT is one of 5 WNT ligands (LIN-44, MOM-2, CWN-1 and CWN-2) in *C. elegans*. It is expressed by cells in the tail of *C. elegans* and creates a gradient from the tail to the mid-body of the animal (Eisenmann, D.M., 2005; Silhankova, M. and Korswagen, H., 2007). It has been shown in *Drosophila* and mammalian models to work either as a repellent or attractant for a diverse number of cell migrations. The characteristic gradient created along the A-P axis suggests that EGL-20/WNT acts as directional cue (Whangbo, J. and Kenyon, C., 1999). Secreted wingless (Wg) proteins, belonging to the WNT family in *Drosophila* can provide cells with positional information; the same role is suggested for EGL-20/WNT in *C. elegans* (Whangbo, J. and Kenyon, C., 1999). The developing gonad primordium in *C. elegans* hermaphrodite also requires WNT signaling to give the two arms their appropriate proximal-distal axis orientation (Eisenmann, D.M., 2005).

A number of different migrating cells depend on EGL-20/WNT for their proper migration during post-embryonic development in *C. elegans*. Among these, the Q

neuroblasts (QL and QR) require EGL-20/WNT for their migration along the A-P axis. Both Q neuroblasts have relatively the same antero-posterior birthplace position. However, their response to EGL-20/WNT differs and results in QL migrating towards the tail and QR towards the head. EGL-20/WNT is responsible for the expression of *mab-5* HOX gene in QL descendents making them migrate posteriorly, unlike the QR descendents that do not express *mab-5* and therefore migrate anteriorly. This makes EGL-20/WNT a permissive signal rather than an instructive one because the response is based on the sensitivity threshold of each Q neuroblast rather than the concentration of EGL-20 (Silhankova, M. and Korswagen, H., 2007). However, Whangbo and Kenyon were able to show that the Q neuroblasts migrate in response to EGL-20/WNT in a dose dependent fashion. High levels of EGL-20/WNT protein induce high levels of *mab-5* expression in the QL descendents which migrate to the posterior while the QR is exposed to low levels of EGL-20/WNT and migrates to the anterior. Furthermore, this group also suggested that this dose dependent response is not triggering the same signaling pathway to different extents but rather is initiating different WNT signaling transduction pathways (Whangbo, J. and Kenyon, C., 1999). The Hox gene *lin-39*, which is a transcription factor in *C. elegans*, is involved in the anterior migration of the QR neuroblast of the pair (QR and QL, QL migrates to the posterior). It establishes regional specificities in the central area of the worm. (Blelloch, R. *et al.*, 1999). Both the WNT and the FGF signaling pathways come together to regulate *lin-39* expression during vulval development (Eisenmann, D.M. *et al.*, 1998).

EGL-20/WNT is also involved in the hermaphrodite specific neurons (HSN) migration to innervate the vulva muscles. The anterior migration of the HSNs in

response to EGL-20/WNT posterior gradient is mediated by the MIG-1 receptor in a cell autonomous fashion. HSN neurons are born in the tail and migrate to the mid-body of *C. elegans* suggesting that EGL-20/WNT acts as a repellent. Mutations of the *mig-1* receptor in HSN cells result in a more posterior position as compared to wild-type suggesting that the repellent effect from EGL-20/WNT is abolished (Silhankova, M. and Korswagen, H., 2007). Also, mis-expression of the EGL-20/WNT gradient in the head of *C. elegans* prevents the HSNs from migrating to their appropriate position and results in a more posterior position along the A-P axis than in wild-type counterparts (Silhankova, M. and Korswagen, H., 2007). It was also shown that other WNT signaling molecules such as LIN-44 and CWN-1 are involved in repelling the HSN during their migration along with EGL-20/WNT. The repelling effect of EGL-20/WNT on HSN anterior migration can be countered by the overexpression of CAM-1, an orthologue of the receptor tyrosine kinase (ROR) (Silhankova M. and Korswagen, H., 2007). This causes the HSN neurons to stop their migration precociously and reside at a more posterior position than the wild-type HSN neurons (Silhankova, M. and Korswagen, H., 2007). Therefore CAM-1 and MIG-1 act as opposing mechanisms on HSN migration.

EGL-20/WNT is not directly involved in the CAN and ALM neuron migrations. However, other WNT ligands, CWN-1 and CWN-2, are critical for the posterior migration of these neurons (Silhankova, M. and Korswagen, H., 2007). They are postulated to act as attractants instead of repellent as seen in the Q neuroblast and HSN migration. Interestingly it is also reported that the migration of CAN and ALM neurons involves the EGL-17/FGF guidance cue (Silhankova, M. and Korswagen, H., 2007).

In *C. elegans* the canonical pathway starts with the WNT ligands (*LIN-44*, *EGL-20*, *MOM-2, CWN-1* and *CWN-2)* signaling through the Frizzled family of receptors (LIN-17, MOM-5, MIG-1 and CFZ-2) which causes the Disheveled proteins (MIG-5, DSH-1 and DSH-2) to inactivate GSK3. This in turn frees β-catenin (BAR-1, WRM-1, HMP-1 and SYS-1) transcription factors which can then translocate to the nucleus to regulate different WNT target genes (Eisenmann D., 2005) (Figure 3). Once in the nucleus, β-catenin can activate POP-1, a TCF/LEF-1 transcription factor, to induce the expression of WNT specific target genes. Without β-catenin activation, POP-1 acts as a negative regulator of transcription through interactions with the Groucho co-repressor (Korswagen, H.C., 2002). The non-canonical WNT signaling can work either through a pathway called the WNT/PCP (planar cell polarity) or the WNT/calcium pathway which are both independent of β-catenin (Eisenmann, 2005; Hoffelen and Herman, 2008). It has been shown that overexpression of WNT proteins and signaling through Frizzled (Fz) receptors has an impact on intracellular calcium levels which impacts on cell response (Veeman *et al.*, 2003). It is thought that the WNT/calcium signaling pathway functions through the same set of Fz receptors as the canonical signaling pathway but diverges after this step. The planar cell polarity signaling pathway was first described in the milkweed bug *Oncopeltus* (Adler, P.N., 2002). It was further characterized in *Drosophila* with the elucidation of the Frizzled signaling pathway and the description of the Frizzled receptors, which are key components of the PCP. PCP rests on the concept that cells usually polarize within the plane of the tissue (proximal-distal axis). For example, the scale and hair found on an animal usually align along the major body axis. In *Drosophila*, the frizzled pathway, through which PCP acts, signals through the Fz

receptor, the disheveled (Ds) cytoplasmic protein, the Van Gogh (Vang) putative membrane protein and Starry night (Stan), a serpentine membrane protein (Adler, P.N., 2002). In *C. elegans*, it has been found that WNT/EGL-20 can signal through a pathway involving Van Gogh/VANG-1 for cell polarity in the developing vulval cells. Interestingly, it was also noted that WNT/EGL-20 signaling through VANG-1 does not involve the Fz receptor as was previously described in *Drosophila* (Green, J. *et al.*, 2008). These non-canonical signaling pathways are still being investigated in *C. elegans* and the number of β-catenin independent pathways is still growing.

The *egl-5* gene is a target gene downstream of EGL-20/WNT signaling pathway (Eisenmann, D.M., 2005). It is one of 4 Hox genes (*ceh-13*, *lin-39* and *mab-5*) in *C. elegans* and is mainly expressed in posterior region, strongest around the anus and tapers off towards the anterior to create a gradient. The expression of *egl-5* is during early larval stages and persists throughout development and is most likely to have a role in giving cells a positional identity (Ferreira, H. *et al.*, 1999).

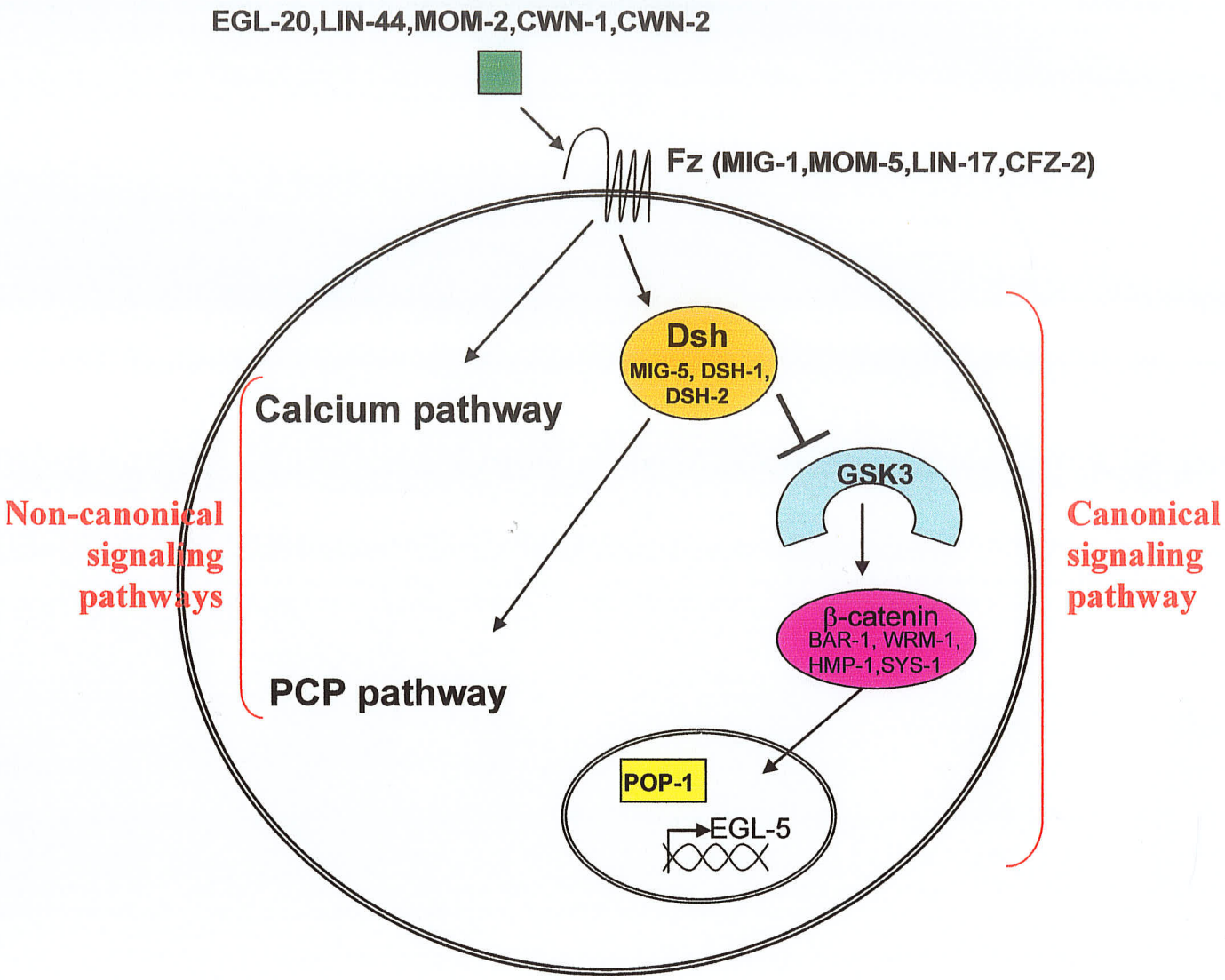


Figure 3. Wnt canonical and non-canonical signaling pathways.
See text for references.

2. RATIONALE

As mentioned in the introduction, cell migration is an important event leading up to development in vertebrates and invertebrates. Cell migration defects are the cause of many developmental disorders and tumor progression. A broader understanding of the multiple pathways interacting for proper cell migration is necessary in order to then address more complex problems such as preventing developmental disorders in developing fetus and halting tumor progression. Studying cell migration in a mammalian model is very complex due to the numerous signaling pathways involved but also the vast numbers of factors and genes that make up these signaling pathways. The *C. elegans* animal model is ideal for this field of study due to the conservation of many of the key signaling pathways that lead to cell migration but also the opportunity to study complex genetic interactions between these elements. The gonad development in *C. elegans* follows an invariant migration pattern and is perfect to study cell migration.

In our research, studying the effects of mutations of elements in EGL-20/WNT and EGL-17/FGF signaling pathways on the DTC migration pattern we can gain insight into how a cell decides to respond to a combination of cues relative to the time and spatial location. Both the WNT and FGF signaling pathways are involved in many developmental processes in the mammalian system and are well known for establishing patterned gradients. Gaining insight into how these different gradients help cells interpret different combinations of signals for cell migration in the *C. elegans* model will help establish basic knowledge of cell migration in the mammalian model.

3. HYPOTHESES

I hypothesize that EGL-20/WNT and EGL-17/FGF have a complementary role in regulating the DTC migration, parallel to that of the UNC-5 cell guidance mechanism. EGL-20/WNT and EGL-17/FGF have an important role regarding the longitudinal (antero-posterior) portion of the DTC migration while UNC-5 is essential for the ventro-dorsal second migration phase. We also hypothesize that these positional cues work alongside temporal cues such as DAF-12 to properly regulate cell migration. Disturbance of one of these signaling pathways may lead to inaccurate spatial and temporal information concerning the DTC position during its migrations.

4. MATERIALS AND METHODS

4.1 Solution and Media preparation

The chemicals used for the experiments described below were purchased through Fisher, Invitrogen, New England Bio Labs, Promega, Sigma, and Qiagen. Solutions and media used for the experiments are described in the solutions section of the appendix.

4.2 *C. elegans* maintenance

Worms were maintained as described in Brenner (1974). Strains were grown on 9 cm or 5 cm plates containing Nematode Growth Media (NGM) agar. Agar was made combining 3 g NaCl, 17 g agar, 2.5 g peptone into 975 ml of ddH_2O along with 1 ml cholesterol (5 mg/ml in EtOH) into a 2 L flask. This solution was autoclaved and then poured into the plates and left to solidify overnight. Any bubbles accumulated in the plates while the agarose solution was being poured were flamed off. *C. elegans* is fed with the *E. coli* strain OP50 obtained through the *Caenorhabditis Elegans* Genetic Centre. This strain is uracil auxotrophic meaning that it is unable to produce uracil and necessitates the supplementation of its media with uracil. This requirement also prevents the overgrowth of *E. coli*. 4 ml of LB (Luria Bertani) broth was inoculated with OP50 *E. coli* culture and grown overnight at 37°C in a shaker and used the next day to seed the agarose plates. Approximately 1ml of the *E. coli* culture was pipetted onto each plate and spread around with a sterile "hockey stick" glass pipette. The plates were left to dry overnight at room temperature before being stored in plastic bins at 4°C till ready to use. *C. elegans* worms were grown on NGM plates with OP50 at 20°C unless otherwise stated.

Worms were transferred weekly from overgrown plates to freshly made ones. This was done using a sterile toothpick to cut a piece of agarose from the old plate and inverting it onto the fresh one so that the worms could crawl off.

In case of contamination, a freshly made bleach solution was used in order to clean the worms. Equal parts of 5 M NaOH and Javex Bleach were mixed in a 15 ml tube and a drop of the solution was deposited onto a clean seeded agar plate. A dozen adult hermaphrodites full of eggs, from the contaminated plate, were picked and transferred into the drop of bleach solution on the clean plate. The adult cuticle was disintegrated by the bleach solution but the robust eggs were left intact. The next day, the clean progeny were carefully picked onto clean agarose plates.

4.3 Long-Term Storage of *C. elegans*

All strains obtained and made in the lab were stored at -80°C. Plates of the worms to be frozen were grown almost to the starvation stage (plate full of worms with little OP50 remaining). Screw-cap freezer tubes were labeled in duplicate and every third sample to be frozen down was done in triplicate. The third sample was taken out of the -80°C the next day to ensure that the worms were still viable. Plates were washed a few times with M9 media and 1ml aliquots were pipetted into the labeled screw-cap tubes along with an equal volume of freezing solution (Brenner, S., 1974). Strain names were entered into the frozen inventory database and tubes were stored in the -80°C, separating the duplicates in different boxes.

4.4 General microscopy

Worms were observed under a Leica MZ6 dissecting microscope for general phenotypical characterization and brood size counts. This microscope was also used when transferring worms from one plate to the next with a platinum wire pick, using aseptic technique. The platinum wire, which was set into the tip of a glass pipette, was flamed till red hot and then cooled down by touching a section of the agar plate devoid of *E.coli* culture. To pick up a worm, the end of the pick was dabbed into thick *E.coli* growth making it sticky and enabling the gathering of *C. elegans* worms with ease. We also used a Zeiss Stemi SV MZBIO Quad 11 for higher magnification to look at worms on plates and to identify GFP positive worms.

4.5 Mutant phenotype analysis

DTC migration errors were readily visualized and quantified as previously described by Headgecock and Culotti (Hedgecock, E.M. *et. al.,* 1990; Culotti and Merz, D.M., 1998). For examples, *unc-5* mutant worms have clear patches at the ventral side of the animal due to the DTC migrating along the ventral side instead of the dorsal side during the third migration phase pushing the intestine aside. A certain number of hermaphrodites from the same brood are examined and the frequency at which the unc-5 phenotype is observed is calculated. The frequency of DTC migration defects can then be compared between different mutant and control strains of *C. elegans* worms.

4.5.1 Live Worm Mounting

Counts of the DTC mutant phenotypes were done on a Zeiss Axio Imager A1 compound microscope equipped with an AxioCam MRc camera. Worms were picked and deposited into 15 µl of M9 on a 2 mm thick 2% agarose pad containing 0.1% sodium

azide. The sodium azide is used in order to immobilize the worms for observation as well as picture photography. The worms were then covered with a 24x24 mm glass cover slip for oil immersion.

4.5.2 Microscopy & Imaging

Worm slides were visualized and counts were made using the 20x and 63x objectives on the compound Zeiss Axio Imager A1 microscope. All pictures were taken with the oil immersion 63x objective (unless stated otherwise), using of the AxioCam MRc camera and processed with Axiovision 6.3 software. Both DIC (Differential Interference Contrast) and fluorescent pictures were taken for GFP strains and overlapped with the use of the software mentioned above. All images of *C. elegans* were oriented such that anterior posterior axis is aligned to the right and dorsal ventral axis with upper and lower respectively.

4.6 Immunofluorescence of worms on poly-lysine slides

Staining was done as described in supplementary online material in Coudreuse. D.Y.M, *et al.* 2006. *WNT Gradient Formation Requires Retromer Function in WNT-Producing Cells*. Science. V312 (921-924). Worms grown up to be healthy and plentiful were washed off into microcentrifuge tubes with M9. In order to clean any OP50 remaining the worms were washed approximately 8-10 times with M9 and spun down at 2000 rpm for 2-3 minutes, removing the supernatant each time. 2-3 final washes with ddH2O followed and after the final spin, the supernatant was removed except for 50 µl. 10-20 µl of worms were deposited and spread out onto poly-L-lysine slides, which were overlaid by a second poly-L-lysine slide. The slides were then placed on a metal sheet over top dry ice for 45 min, in order to freeze crack the cuticles of the animals. Once the

slide sandwich was cracked, slides were immersed in ice-cold methanol followed by acetone for 4 minutes each. The worms were rehydrated in 90, 60 and 30% ethanol in PBS and then PBS alone for two minutes each. Slides were then incubated in skim-milk blocking solution for an hour rocking slowly at room temperature. Staining was done by laying the slides out in a tupperware container lined with a damp cloth. 75-80 µl of 1/500 dilution of antibody was pipetted onto the slides and incubated for an hour at room temperature in the dark. The slides were once again incubated in the blocking solution for one hour, gently rocking at room temperature in the dark. Before sealing, the slides were incubated in PBS for 5 minutes and then laid out on a dry paper towel. 20 µl of mounting medium was placed over the worms and a 24 x 60 mm cover slip was laid overtop followed by sealing the slides with 2-3 coats nail polish. The slides were stored at 4°C in the dark for days to a few weeks.

4.7 β-galactosidase staining

Fresh paraformaldehyde solution was made for every staining experiment. In a 1.5 ml Eppendorf tube, 200 mg of paraformaldehyde was mixed with 900 µl of 5mM NaOH and heated in a 60°C water bath for 15 minutes. The solution was immediately centrifuged and the supernatant was used as indicated.

Worms to be stained were washed with 1-2 ml of M9 solution and transferred to a 1.5 ml Eppendorf tube. They were left to settle and the supernatant was discarded. A fresh batch of MRWB (modified Ruvkun's witches brew) was also made for every staining experiment from a 4x stock kept at 4°C. 400 µl of ddH_2O along with 250 µl cold 4x MRWB, 250 µl cold methanol and 100 µl paraformaldehyde solution (mentioned above) were combined. 900 µl of this solution was then added to the Eppendorf tube containing

the worms to be stained, mixed and put on ice for at least 35 minutes with occasionally mixing.

After the incubation period the worms were left to settle and the supernatant was discarded. TTB was used to wash the worms twice, leaving the worms to settle between each wash, followed by incubation in TTB (Tris Triton Buffer) at room temperature for an hour. The final wash was done with ddH_2O, once again leaving the worms to settle before removing the supernatant.

The staining was done by adding 620 µl H_2O, 250 µl of 0.8 M Na phosphate (pH 7.5), 2 µl 1.0 M $MgCl_2$, 4 ul 1% SDS, 100 µl redox buffer (equal parts of 100 mM potassium ferricyanide and 100 mM potassium ferrocyanide) and 20 µl 2% X-Gal to the treated worms and incubating at 37°C for 1-2 hours (Su, M. *et al.,* 2000). Stained worms were washed once more with ddH2O before pipetting them onto a 2% agarose pad on a slide. Worms were then covered with a 24x24 mm cover slip for analysis under the compound microscope.

4.8 Statistical Analysis

Worms were counted to obtain approximately N=100 for each strain observed. Statistical analysis comparing the proportions of wild-type and mutant worms was done using Chi square analysis. A minimum of 0.05 critical value was used to find statistically significant results. Yates correction was applied where necessary. The SigmaStat 2.0 software was used to do the statistical analysis

4.9 *C. elegans* Strain List

Strain name (genotype)	Source
unc-5GFP	This work
unc-5(ev585)	Merz
unc-5(ev585); unc-5GFP	This work
egl-17(e1313)X	CGC
egl-17(e1313)X; unc-5GFP	This work
egl-20(n585)	CGC
egl-20(n585); unc-5GFP	This work
egl-20(n585); egl-17(e1313)X; unc-5GFP	This work
mpk-1(ku1) unc-32(e189)III	CGC
mpk-1(ku1) unc-32(e189)III; unc-5GFP	This work
unc-32(e189)III; unc-5GFP	This work
daf-12(rh411)X	Merz
egl-17(e1313)X daf-12(rh411)X; unc-5GFP	This work
egl-20(n585); daf-12(rh411)X; unc-5GFP	This work
lin-17(n671)I	CGC
lin-17(n671)I;egl-17(e1313)X;unc-5GFP	This work
lin-17(n671)I; unc-5GFP	This work
egl-15(n484)	CGC
egl-20(n585); egl-15(n484); unc-5GFP	This work
egl-15(n484); unc-5GFP	This work
lin-25(n545)V	CGC
lin-25(n545)V; unc-5GFP	This work
egl-20(n585)IV;lin-25(n545)V; unc-5GFP	This work
dpy-22(sy622)X	CGC
dpy-22(sy622)X; unc-5GFP	This work
egl-20(n585)IV; dpy-22(sy622)X; unc-5GFP	This work
clr-1(e1745)II	CGC
clr-1(e1745)II; unc-5GFP	This work
clr-1(e1745)II; egl-20(n585)IV; unc-5-GFP	This work
egl-5(n945)III	CGC
egl-5(n945)III; unc-5GFP	This work
egl-5(n945)III; egl-17(e1313)X; unc-5-GFP	This work
mig-1(e1780)I	CGC
mig-1(e1780)I; unc-5GFP	This work
mig-1(e1780)I; egl-17(e1313)X; unc-5-GFP	This work

lin-15AB(n309)X	CGC
lin-15AB(n309)X; unc-5GFP	This work
egl-20(n585); lin-15AB(n309)X; unc-5GFP	This work
egl-20(n585); egl-17(e1313)X lin-15AB(n309); unc-5GFP	This work
sdn-1(ev697)	Schwabiuk
sdn-1(ev697);egl-20proteinA	This work
sdn-1(zh20)	Rhiner et al.
sdn-1(zh20);egl-20proteinA	This work
egl-20proteinA	Coudreuse

Table 1. *C. elegans* strain table
Most double and triple mutants were obtained by crossing two different mutants together (see section 9.2 of the appendix for further information on crossing strains).

4.10 Mutant strain generation

In order to properly score the DTC migration defects, we used a reference point in the worms expressing *unc-5* tagged with GFP (*unc-5GFP*). Expression of this protein was seen not only in the DTC but also along the ventral nerve cord in motorneurons. The third doublet of the motorneuron cell bodies, from the posterior of the worm, is made up of VD10 and DD5 ventral neurons and represent the reference point over which the second migration phase occurs in wild-type worms. Therefore many of the strains mentioned include *unc-5GFP*. To do this the strain of interest was crossed with males from the *unc-5GFP* strain (4 males: 1 hermaphrodite) and let to mate and reproduce for 3 days. Plates were checked for any male progeny which indicates a successful cross and proceed to "picking wild-type looking worms". Wild type worms displayed to apparent DTC migration defects under the dissecting microscope (no clear patch like the Unc-5 phenotype) and did not display any of the other mutant phenotypes being crossed (ex: DPY mutations give the worm a short stubby stature. A wild-type looking worm would be of the normal size, relatively long and slender). The successful cross is then put under

the fluorescent light to identify worms that contain the GFP tag and are picked onto a separate plate to maintain this phenotype.

4.11 RNAi Knockdown Experiment

4.11.1 RNAi Feeding Protocol

RNAi feeding was done as described by the Ahringer lab protocol. RNAi plasmids were chemically transformed into HT115 *E. coli* Tet^R cells which were plated onto LB plates containing ampicillin and tetracycline to select for transformed cells (NEB transformation protocol). The plates were grown up overnight at 37°C. Colonies were picked to make 4ml LB liquid cultures containing only ampicillin, grown overnight at 37°C. NGM plates containing 25 µl/ml Carbenicillin and 1mM IPTG were seeded with the 4 ml grown up cultures. These plates were left to dry for a few days and developed a thick lawn of *E. coli* before putting 2-3 worms on them to observe the effect of the RNAi gene knowckdown.

4.11.2 Preparation and transformation of chemically competent cells

HT115 cells are used to produce the RNAi strands that are fed to *C. elegans* in order to knockdown specific genes. Cultures from the -80°C were spread onto LB Tet plates and incubated overnight at 37°C. The Tetracycline is selective for the growth of HT115 as it is Tet resistant. Single colonies were picked the next day and used to inoculate 4ml cultures containing 4ul of Tet (final concentration of 12.5 µg/ul) which were grown overnight at 37°C in the shaker. Some of the HT115 bacterial cultures were frozen down for future use. 0.6 µl of glycerol (20% final concentration) was added to 0.9 µl of culture in sterile screw-cap freezer tubes and put on dry ice for 10min for rapid freezing before being stored in the -80°C.

The remaining HT115 cultures were used to make chemically competent cells. 1 ml of the cultures was used to inoculate 100 µl LB Tet and put in the 37°C shaker for approximately 3-3.5 hours. Every hour we checked the concentration of the bacterial growth until we obtained an $OD_{595nm} = 0.4$. The culture was then aseptically transferred into sterile, disposable, ice-cold 50ml polypropylene tubes and stored at 0°C for 10 min. The cells were then centrifuged for 10min at a speed of 4000 rpm at 4°C after which the supernatant was decanted. Tubes were inverted onto paper towels for 1min in order to get rid of the remaining growth media. The pellet was resuspended with 10ml of sterile, ice-cold 0.1M $CaCl_2$ and then incubated at 4°C for 10min. Cells were once again spun down at 4000 rpm at 4°C for 10 min and the supernatant decanted. Pellets were resuspended a second time in 2 ml of ice-cold 0.1M $CaCl_2$ and transferred into sterile centrifuge tubes in aliquots of 200 µl. At this point cells could either be transformed with the plasmid of interest or frozen for future use. Cells put in the -80°C freezer did have a reduced transformation efficiency compared to the freshly treated cells. Glycerol was added to a final concentration of 10% to the $CaCl_2$ treated cells to be stored. They were then rapidly frozen in a dry-ice bath for 10min before being stored in the -80°C.

Competent cells to be transformed were inoculated with 1µl of plasmid DNA (1pg-100 ng) and then placed on ice for 30min. The cells were then heat shocked at 42°C for 90sec and then immediately placed on ice once again for 5 min. After incubation, the cells were transferred to a sterile 15ml culture tube and 950 µl of room temperature SOC was added. The cultures were put into a 37°C shaking incubator for 45min after which they were spread onto pre-warmed LB Ampicillin and Tetracycline plates (see Appendix) in 50, 100 and 200 µl aliquots and spread with a sterile glass rod. The plates were incubated

overnight at 37°C. Colonies growing on the plates are an indication of a successful transformation as the cells have acquired the ampicillin resistance from the plasmid DNA introduced into the competent cells. Theses colonies were then used to inoculate 4 ml LB Amp cultures that were be grown overnight at 37°C and used to inoculate LB Amp, Carbenicillin and IPTG containing plates for RNAi induction.

The first plasmid DNA used for RNAi induction were *ptp-2* plasmid 4B04 II along with the empty plasmid vector pPD129.36 .

4.11.3 Gel electrophoresis

DNA samples were run in a 1% agarose gel to verify that the appropriate constructs were used. 0.2 g of agarose (Promega) combined with 20 ml of 1X TAE (Tris-acetate-EDTA) buffer were melted for 30 seconds in a microwave till the agarose was completely dissolved. 2ul of 10mg/ml ethidium bromide (EtBr) stock was added (10% of total TAE volume) to the hot solution before pouring into a 100x115 mm casting mold with the appropriate comb in place (combs have varying well numbers). The EtBr is necessary to track and visualize the DNA strands once they are run in the gel matrix when put under ultraviolet light. Once the gel was solidified, it was submerged in 1X TAE solution and the comb was be taken out. 2 µl of 10X OrangeG dye was added to 10 µl of each sample (1:5 ratio) after which the samples were loaded into the wells along with an appropriate ladder. 10 µl of 100 bp ladder can be used depending on the size of DNA fragment expected to be run. The OrangeG dye is necessary in order to track the progress of the DNA as it is being run along the gel. A current of 80-90 volts (Fisher FB300 power pack) was applied for 30-45 minutes or until the DNA bands had sufficiently separated on the gel. The gel was then taken out of the mold and DNA bands were visualized

under AlphaImager2200 trans-illuminator and a picture was taken using the AlphaEaseFC software.

4.12 Cellular Locus of Signaling

To identify the critical location of cell signaling we resorted to using an *rde-1* mutant strain, which is necessary for RNAi-mediated interference (Tabara, H., *et. al.,* 1999). pMR541 is a promoterless *rde-1* cDNA construct made up of the *rde-1* sequence including the initiator methionine inserted into the pPD49.26 plasmid (kind donation from Dr. Richard Roy at McGill University, Montreal). Any promoter can be inserted upstream of the coding sequence in order to create a tissue or cells specific rescue construct. The *lag-2* promoter drives transgene expression in the DTCs of *C. elegans.* Promoters were designed to include 1.8 kb upstream of the *lag-2* start methionine as well as a BamH1 cut site at either end (F-BamH1.2 5'tctgttac***ggatcc***cttgctctgcgacgagc and R-BamH1.2 5'caggtgaggagtaagaggaa***ggatcc***gatcct (bold indicates the BamH1 cut site and italic indicates the mutated nucleotides)). Both the vector and primer were digested with BamH1 and only the vector was treated with CIP (calf intestinal phosphatase) before being ligated to create PLC3 and transformed into bacterial culture. Directionality of the insert was verified by restriction enzyme digest using SacI (single digest) and PstI and SacI (double digest). A GFP construct of a similar nature was used as a positive control. pPD95.69 is a promoterless GFP vector into which we cloned the *lag-2* promoter, this time using a different set of primers. The forward primer is 1.9Kb from the methionine start site of *lag-2* gene and includes an engineered HindIII cut site (F-HindIII.3 5'ctcctaatacc***aagctt***catgcctttg). The reverse primer was designed to have its Sph1 cut site directly after the *atg* initiator methionine (R-lag2-Sph1.2

5'gagtaagaggaagtaagcga***gcatgc***tctg (bold indicates the HindIII and Sph1 cut site respectively and italic indicates the mutated nucleotides)). This positive control confirms that the promoter region used to drive both constructs is sufficient to drive expression specifically in the DTC.

4.13 Nematode Genomic DNA Isolation

Worms were grown on Rich Agarose Plates (see Appendix) until just before the starvation phase. They were harvested by washing the plates with dH_2O and collected into 15 ml tubes and subsequently spun for 30 sec at 500 rpm. The pellet was washed twice with dH_2O to remove any residual bacterial culture after which worms were resuspended in 1 ml dH20 and transferred to a 1.5 ml eppendorf tube. For the worm lysis, the worms were spun down and resuspend in 500 ul of worm lysis solution (see Appendix) and put at -80°C for at least 30 min. Next the worms were incubated at 55-65°C for another 30-60 min, agitating occasionally. The solution was transferred to a new tube without transferring the large debris. To clean up the solution, the solution was treated with equal volumes (~500 ul) of phenol/chloroform and centrifuged for 1min at maximum speed. The aqueous phase was extracted and treated with an equal volume of phenol/chloroform once again and spun the solution for 1min at 13,200 rpm. If needed, the extraction was repeated to further clean up the aqueous phase. The DNA was then precipitated by adding 2.5 volumes of ice-cold 99% EtOH to the aqueous phase and centrifuge for 10 min at maximum speed. This step was twice more repeated using ice-cold 70% EtOH and spinning after each wash for 10min at maximum speed. The Eppendorf tubes were inverted on a paper towel to air dry before resuspending. Genomic DNA was resuspended with 750 ul of low TE.

5. RESULTS

5.1 *sdn-1* mutants have aberrant function of EGL-20/WNT and EGL-17/FGF

In a genetic screen, enhancers of the DTC migration defects of an *unc-5* hypomorph were identified. One genetic enhancer identified was *sdn-1* which encodes the *C. elegans* homologue of Syndecan. The *sdn-1* mutant gene enhanced the DTC migration defects as it displayed an increased penetrance of the Unc-5 phenotype in double mutants of *unc-5(e152); sdn-1(ev697).* SDN-1 is a trans-membrane proteoglycan with a modified heparan-sulfate side-chain (Minniti, A.N. *et al.*, 2004). Previous work has shown that proteoglycans have an active role in DTC guidance (Merz, D.M. *et al.*, 2003) and that SDN-1 does not act within the DTC to guide it during the migration phases but rather acts in a cell non-autonomous manner (Schwabiuk, M., 2006). It is known that membrane bound heparan sulfate proteoglycans such as syndecan interact with a variety of signaling pathways such as TGFβ, WNT, EGF and FGF during development (Baeg, G.H. and Perrimon, N., 2000) and axon guidance (Charron, F. and Tessier-Lavigne, M., 2005). It is proposed that SDN-1 acts to limit and/or localize growth factors required for the proper migration of the DTC (Schwabiuk, M., 2006). Mutations of *egl-20* and *egl-17* were shown to mask the effects of *sdn-1* mutations on the DTC migration. Coudreuse *et al.* (2006) had previously demonstrated the graded distribution of EGL-20/WNT using a *protein-A* reporter construct and immunostaining with swine-anti-goat-FITC antibody (Coudreuse, D.Y. *et al.*, 2006). A negative control of the wild-type without the *egl-20protein-A* tag was done and did not display any immunostaining after treatment with the immunoglobulins (Figure 4A). Using this same technique, we were able to show the normal distribution of *egl-20protein-A* being secreted by the cells in the tail of

hermaphrodites and diffusing out in wild-type worms (Figure 4B). The *sdn-1(ev697)* allele encodes a premature stop codon preceding the trans-membrane domain (Rhiner *et al.* 2005). We believe that this mutation results in the mutants expressing an unbound form of SDN-1 (Schwabiuk, M., 2006). In *sdn-1(ev697)* mutant allele we consistently observed a more anterior distribution of EGL-20/WNT along the A-P axis of *C. elegans* as well as increased expression of *egl-20* compared to wild-type (Figure 4C). This can be explained by the fact that the premature stop codon probably secretes a SDN-1 protein that is not anchored. It therefore is not capable of maintaining the EGL-20/WNT protein gradient in the tail and spreads the expression to a more anterior position than in wild-type. The *sdn-1(zh20)* single mutant is a presumed null allele. This suggests that the SDN-1 protein is absent and in turn is unable to bind and properly distribute EGL-20/WNT along the A-P axis of the worm as seen in wild-type worms. Repeated experiments of the immunostaining of *sdn-1(zh20)* also displayed an increase expression level as in *sdn-1(ev697)* (Figure. 4D). These results suggest that SDN-1 has a role in regulating the expression level and localization of EGL-20/WNT. Given these results we decided to further investigate the involvement of the EGL-20/WNT and EGL-17/FGF in migration of the DTCs.

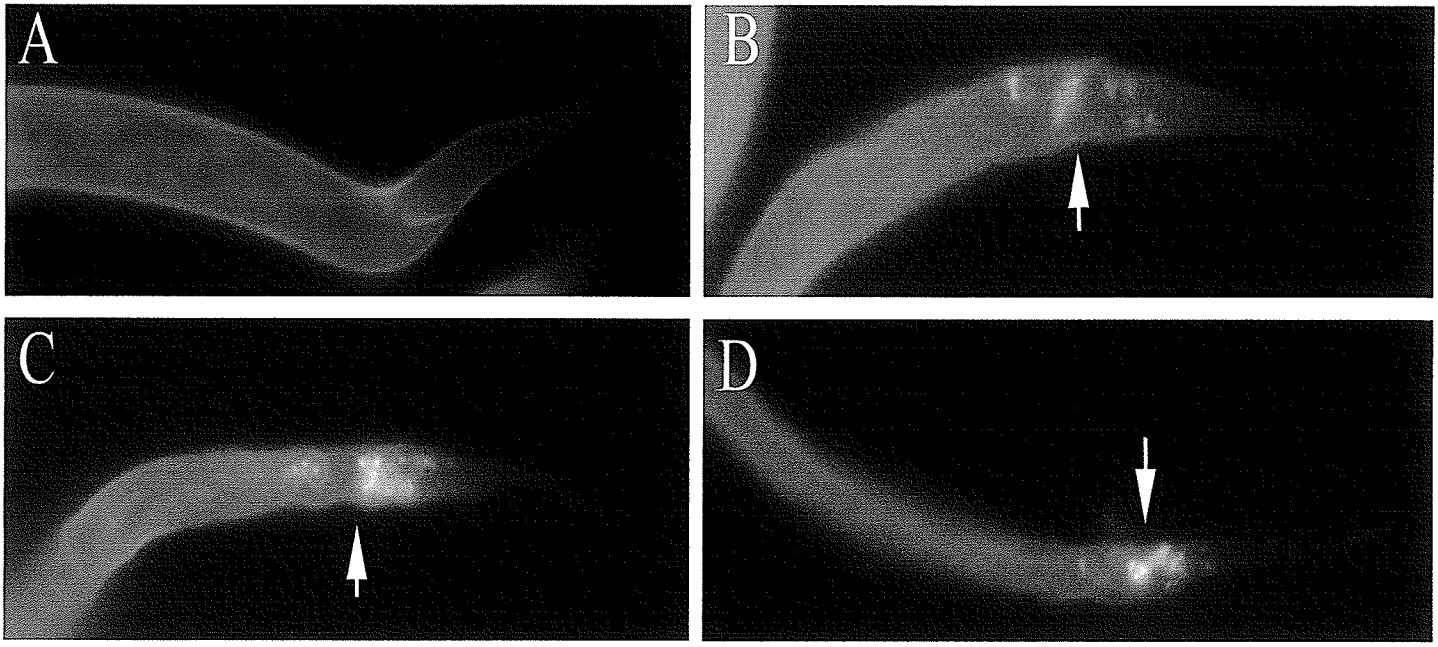


Figure 4. *sdn-1* mutants display a more anterior distribution of EGL-20/WNT along with an increased expression of this protein.
Fluorescent pictures of the tail of four different *C.elegans* strains. The worms were stained with *protein-A* antibody to detect the expression level and distribution of EGL-20/WNT in the tail. **A** represents the negative control for the experiement. EGL-20/WNT is not tagged with *protein-A*. **B** is the positive control where EGL-20/WNT was tagged with *protein-A* in a wild-type strain. We can observe the appropriate expression level and distribution of EGL-20/WNT being secreated by cells in the tail of the worm. **C** is *sdn-1(ev697);egl-20;protein-A*. This SDN-1 allele has a premature stop codon which presumably produces a protein without the transmembrane domain. This results in mis-distribution of EGL-20/WNT protein as can be seen in **C**, where EGL-20/WNT has a more anterior distribution and overexpression than the wild-type as shown by the white arrows. *sdn-1(zh20);egl-20-protein-A* (**D**) represents a null allele of SDN-1 and therefore EGL-20-protein-A is only located in the cells at the very end of the tail. The usual gradient along the posterior of the worm appears to be abolished.

5.2 *egl-20; egl-17* double mutants display increased DTC migration defects.

Since we proposed from the above data that EGL-20/WNT and EGL-17/FGF had a role in regulating the DTC migration, we decided to further characterize the different migration defects in these mutants. Mutant strains were generated (see section 9.2) and then scored using still worm microscopy and imaging with a compound microscope.

We specifically observed the migration pattern of the posterior DTC and gonad arm in *C. elegans* hermaphrodites. In order to compare one worm to the next we selected a ventral neuron doublet, VD10 and DD5 (identified by a double asterisk in Figure 5), which expresses an *unc-5GFP* reporter construct. The reference point was selected by observing wild-type worms and observing where (along the ventral side of the animal) the second migration phase was initiated. The reporter construct is also useful to identify the DTC, which expresses *unc-5* during the second migration phase, and also makes the VD10 and DD5 doublet readily visible since it expresses GFP throughout most of *C. elegans* developmental stages. When looking at animals, we took care to select worms based on age, since we only wanted to observe gonad phenotypes in late L3 to late L4 worms. This was necessary since fully grown adult hermaphrodites could display misleading phenotypes due to the size of the gonad arm and the development of eggs. Using this protocol we proceeded to observe how the DTC migrations differed in either the *egl-20* or *egl-17* single mutants or *egl-20; egl-17* double mutants compared to wild-type worms.

Single mutants of *egl-17* displayed few migration defects (94% wild-type, see Table.1) in all the 49 worms observed, comparable to wild-type worms. The remaining 6% of the *egl-17* single mutant worms displayed first migration defects: 4% and 2%

precocious and late, respectively, but these defects were not found to be significant. Single mutants of *egl-20* displayed third phase longitudinal migration defects (12%, N=56) compared to their wild-type counterparts. Third phase longitudinal migration defects were characterized by the posterior DTC failing to return to the mid-body during the third migration phase and instead migrating towards the tail region (Figure 5C). In *egl-20; egl-17* double mutants we observed a significant increase in the frequency of DTC migration defects concerning the first phase precocious migration defects (20%, N=64). However the frequency of the third phase longitudinal migration defects (14%, N=64) was not significantly different from that of the *egl-20* single mutants. Interestingly, the composition of the third phase migration defects did change when comparing *egl-20* single mutants to *egl-20; egl-17* double mutants. The single mutant presented a majority of third migration defects referred to as 'zigzag' whereas the double mutants displayed very few worms of this phenotype. The 'zigzag' phenotype consisted of the DTC starting to migrate back to the mid-body and then turning back into the tail (see cartoon drawing in Table 1.) In the double mutants the third phase migration defects were almost exclusively longitudinal without any hesitations from the DTC. First phase precocious migration defects were characterized by the posterior DTC initiating the second migration phase at a more anterior position along the A-P axis in relation to the reference ventral neuron doublet VD10 and DD5 (Figure 5D). Since we only observed a few defects in the single mutants compared to the significant difference in DTC migration defects in *egl-20; egl-17* double mutants, we can suspect that EGL-20/WNT and EGL-17/FGF signaling pathways have overlapping/redundant roles in regulating the

DTC migration during this phase and that they might regulate both A-P axis migration phases.

			First phase		Third phase	
Strains/ Phenotypes	N	wild-type	precocious	late	zigzag	longitudinal
unc-5GFP (wild-type)	114	93.00%	7.00%	0.00%	0.00%	0.00%
egl-17;unc-5-GFP	49	94.00%	4.00%	2.00%	0.00%	0.00%
egl-20;unc-5GFP	56	88.00%	0.00%	0.00%	10.00%	2.00%
egl-20;egl-17;unc-5GFP	64	59.00%	20.30%	3.10%	3.10%	14.10%

Table 2. *egl-20; egl-17* double mutants display increased DTC migration defects. Table represents the percentage of the total N number of worms belonging to each category for each strain. Gonad morphology was observed under the compound microscope and DTC migrations were counted and then classified based on the migration pattern presented in each worm. Both *unc-5-GFP* and *egl-17;unc-5-GFP* do not present any significant migration defects whereas *egl-20;unc-5*-GFP presents some longitudinal migration defects. The double mutant *egl-20; egl-17;unc-5*-GFP has a marked increase in the number of longitudinal defects but also in the precocious migration of the DTC.

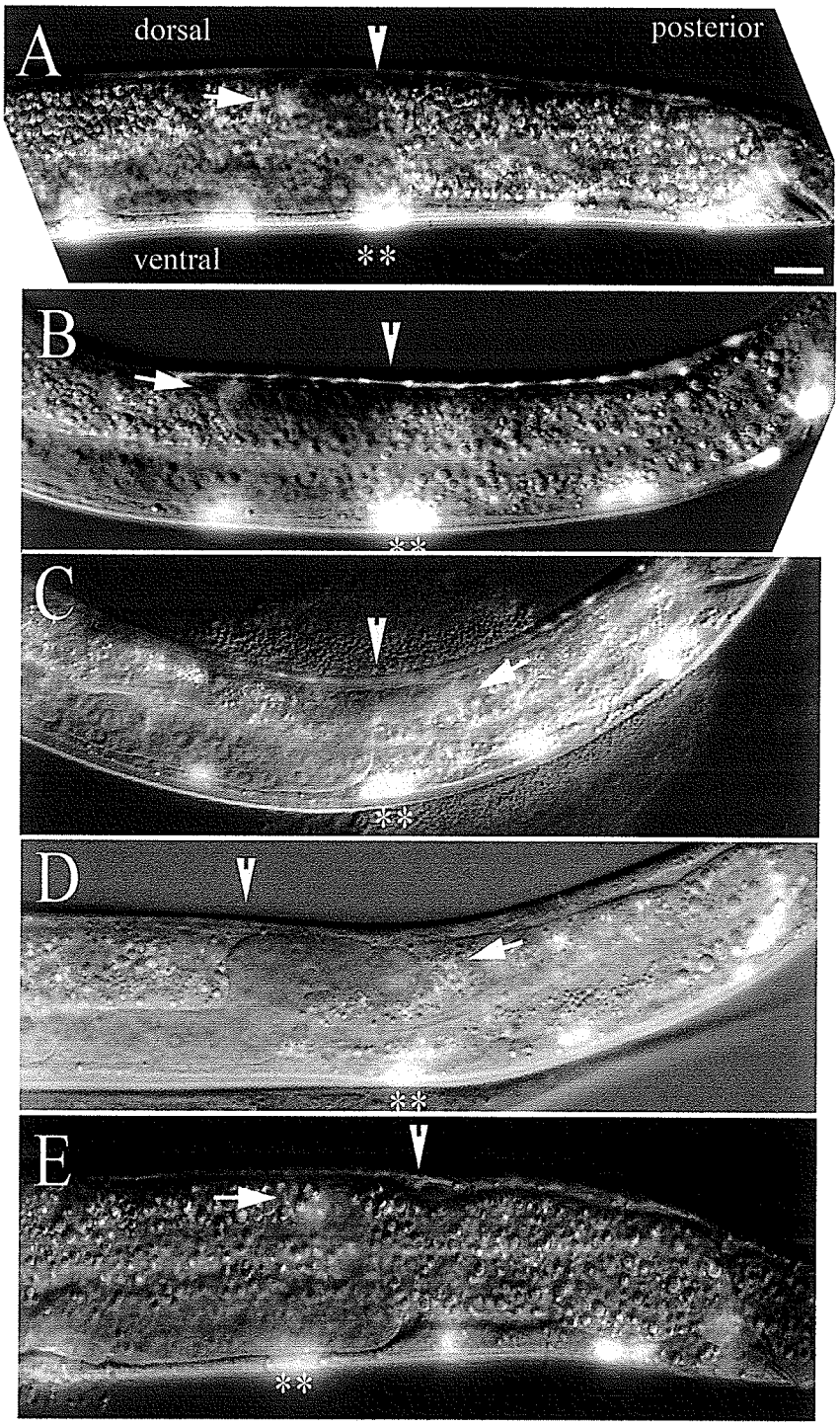

A
dorsal
posterior
ventral
B
C
D
E

Figure 5. *egl-20; egl-17* double mutant display increased DTC migration defects. Overlay of DIC and fluorescent pictures of *C.elegans* posterior gonad arm. Worms are all positioned facing the left side (tails are to the right) and the ventral side is downward (dorsal side is upward). **A** *unc-5GFP* (wild-type) and **B** *egl-17;unc-5GFP* single mutant do not display any distal tip cell (DTC) migration defects. **C** *egl-20;unc-5GFP* single mutants display some third phase longitudinal migration defects characterized by the DTC failing to come back to the dorsal mid-body during the last migration phase. **D** *egl-20; egl-17;unc-5GFP* double mutants also display third phase longitudinal migration defects along with first phase precocious migration defects. The precocious migrations are characterized by the DTC initiating the second migration from ventral to dorsal at a more anterior position along the A-P axis. **E** First phase late migration defects was occasionally seen in mutants of components of EGL-20/WNT and EGL-17/FGF signaling pathways such as *clr-1* mutants. The double asterisks indicate the ventral neurons VD10 and DD5 used as a reference points over which the DTC normally turns from ventral to dorsal during the second migration phase in wild-type animals (**A** and **B**). Arrowheads indicate the position of the DTC and arrows designate the position along the A-P axis where the DTC turned form ventral to dorsal (second migration phase).

5.3 EGL-20/WNT and EGL-17/FGF do not regulate *unc-5* transcription.

To better determine if the DTC is turning at an earlier developmental stage we examined whether EGL-20/WNT and/or EGL-17/FGF regulate the pattern or timing of *unc-5* expression. Up-regulation of *unc-5* is important for the second migration phase. Expression of *unc-5* drives the repulsion away from UNC-6 resulting in the DTC migrating from the ventral to dorsal sides (Su, M. *et al.*, 2000). Precocious expression of *unc-5* results in a corresponding dorsal migration of the DTCs (Su, M. *et al.,* 2000) which are very similar to the *egl-20; egl-17* phenotype. Since we had observed precocious V-D migration of the DTC along the antero-posteiror axis, we hypothesized that EGL-20/WNT and EGL-17/FGF may have a role in regulating the expression of *unc-5* at the transcriptional level.

Initially we used a GFP reporter construct to monitor the expression of *unc-5* in the DTC. When observing the DTC migration we were unable to observe the earliest expression of *unc-5GFP*, when the DTC turns from the ventral side towards the dorsal side, and were only able to detect *unc-5GFP* in the DTC during the second or third migration phase. This resulted from the lag time between the transcription and translation of GFP being too long (more than one hour) and preventing us from accurately detecting the earliest expression *unc-5* (Su M. *et al.*, 2000).

To address this problem, we used a Lac-Z reporter construct which was more accurate in detecting the expression of *unc-5* in the DTC (Su M. *et al.*, 2000). To observe worms using Lac-Z reporter constructs, worms were fixed and stained as described in section 4.7, and were then observed as described in section 4.2. Wild type worms (*unc-5Lac-Z)* displayed appropriate timing and synchronized expression of *unc-5* in the DTCs. We

were successful in observing *unc-5* expression in the very early stages of the second migration phase in both the anterior and posterior DTCs. In the double mutants (*egl-20; egl-17; unc-5Lac-Z*) worms, we observed normal developmental timing of *unc-5* up-regulation during the second migration phase regardless of the posterior DTC positioning along the antero-posterior axis (Figure 6). More precisely, the anterior and posterior DTCs were not migrating in a synchronized matter. When the anterior DTC reaches the normal turning point, the posterior DTC has not yet reached its normal turning point. However both DTCs were simultaneously expressing *unc-5LacZ*. This came as a surprise since we expected the posterior DTC to be turning earlier in development.

We expected to see *unc-5LacZ* expression in the posterior DTC but not in the anterior DTC in double mutants of *egl-20; egl-17*. This scenario would have indicated that both EGL-20/WNT and EGL-17/FGF have a role in regulating the transcription of *unc-5* in the posterior DTC to initiate the second migration phase. Instead we observed that *unc-5* expression was not altered the *egl-20; egl-17* double mutant and the initiation of expression independent of the position of the posterior DTC. Together this data suggests that the posterior DTC is starting its first migration phase at a later time or is migrating at a slower rate along ventral side of the worm in the *egl-20; egl-17* double mutants. Therefore *unc-5* is not directly regulated at the transcriptional level by EGL-20/WNT and EGL-17FGF.

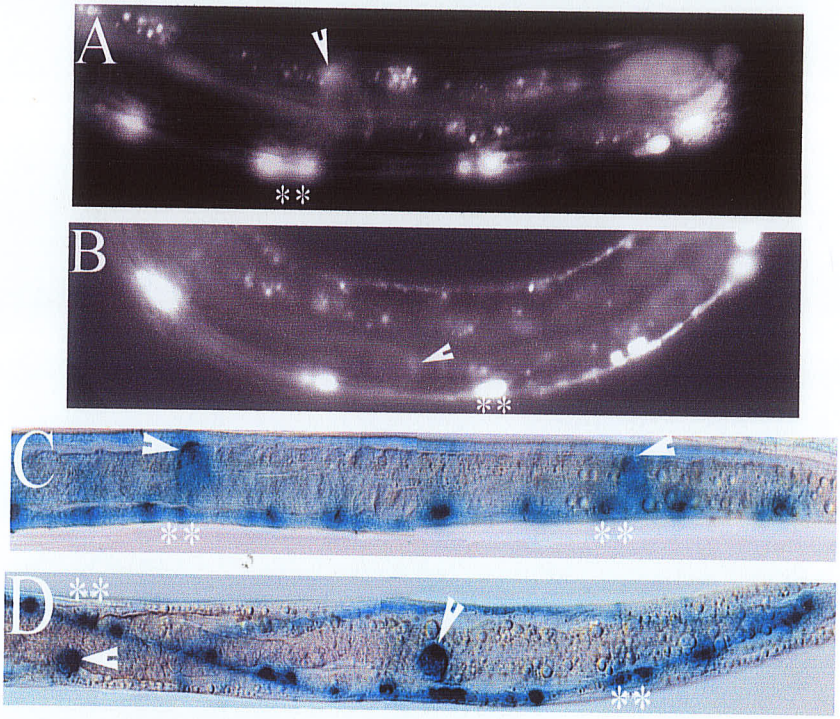


Figure 6. EGL-20/WNT and EGL-17/FGF do not regulate *unc-5* expression at the transcriptional level.
A and **B** are fluorescent pictures of the posterior gonad arm while **C** and **D** are LacZ staining of both the anterior and posterior DTCs of *C.elegans* hermaphrodite worms. **A** and **C** represent the wild-type strain, where the DTC turns over the reference point VD10 and DD5 (double asterisks) and is expressing *unc-5* (either tagged with GFP or LacZ respectively). **C** shows that both the anterior and posterior DTCs completing their second migration phase and both DTCs have turned over thier respective reference points (double asterisks). Both DTCs are migrating at a synchronous rate and are expressing *unc-5 LacZ*. **B** and **D** represent *egl-20; egl-17* mutant worms where the posterior DTC displays *unc-5* expression regardless of its position along the A-P axis of the worm. **D** shows the anterior DTC which has completed the second migration phase and is starting the third along the dorsal side (the worm is twisted and so the anterior part of the worm has the ventral side facing upwards and the dorsal side facing downwards). Meanwhile, the posterior DTC is lagging behind and is still completing the first migration phase along the ventral side. Both the anterior and posteior DTCs are expressing *unc-5LacZ* indicating that mutations of *egl-20* and *egl-17* do not affect the transcription of *unc-5* during the second migration phase.

5.4 *egl-20; egl-17* double mutants posterior DTC moves at a slower rate relative to vulva development.

Initially we attempted time lapse experiments in order to track the migration of the posterior DTC over time and compare DTC migration rates in different mutant backgrounds. After many attempts with different solutions to paralyze the worms without killing them, we were unable to sustain viability long enough to capture a series of pictures for this purpose. Instead we decided to compare gonad development with an independent developmental process in *C.elegans*; the development of the vulva. We were able to observe parallel trends between the gonad development and vulval development which helped to track the appropriate developmental timing of both events. We started out by looking at wild-type (*unc-5GFP*) worms with DTCs completing their second migration phase (when the DTC reaches the dorsal side of the animal). At this developmental stage (when the DTC has reached the dorsal side) we observed that the vulval cells are pyramidal and are preparing to invaginate to form the adult vulva (Figure 7. A & Ai). To ensure consistency, we also observed worms with DTCs at earlier and later migration stages and observed their developing vulvas as well. We were able to determine that both the vulva and the gonad develop in a consistent and parallel manner.

In wild-type (*unc-5GFP*) worms, we observed that the vulva has not yet started to invaginate when the DTC had reached the dorsal side of the animal and is entering its 3rd migration phase (Figure 7A&Ai). In contrast, the double mutant *egl-20; egl-17*worms displayed much more mature vulvas at a comparable stage of DTC migration (Figure 7B&Bi). In contrast to wild-type worms where the vulval cells are in a pyramid shape the double mutant vulva has already started to invaginate to create the adult vulva. Assuming that the vulva is developing at a normal rate, the posterior DTC is migrating at

a slower rate during the first and second phases. The vulva is therefore much more developed by the time the posterior DTC enters the second migration phase compared to the wild-type.

This finding further supports our hypothesis that the posterior DTC is migrating at a slower rate along the A-P axis in *egl-20; egl-17* double mutants. This results in the initiation of the V-D migration phase at the correct time but at a more anterior position.

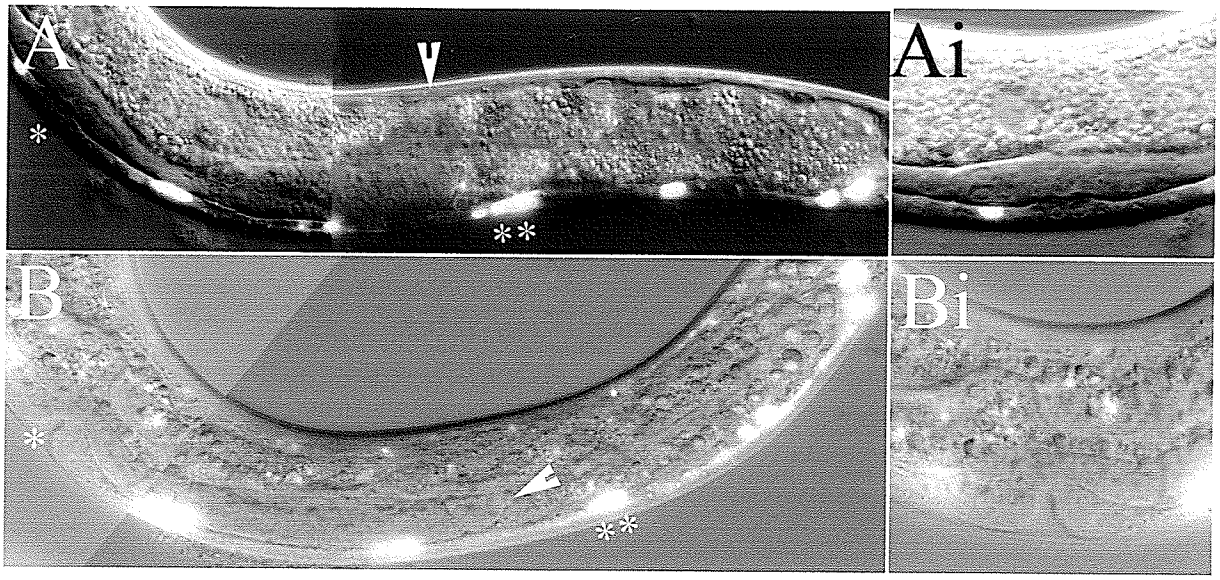


Figure 7. *egl-20;egl-17* double mutants DTC moves at a slower rate relative to vulva development.

Pictures of posterior DTC migration in wild-type *unc-5GFP* (**A**) and double mutant *egl-20;egl-17;unc-5GFP* (**B**). In the wild-type worms the DTC in entering its 3rd migration phase when the vulva is about to invaginate (**Ai**) whereas in the double mutant the vulva is well developed (**Bi**) even before the DTC has entered its second migration phase (**B**). Panel **A** is a DIC picture while **B** is a DIC and fluorescent overlay. Arrowheads indicate the position of the DTC along the A-P axis and single asterisk indicate the positioning of the vulva. Double asterisk in panel **B** depicts the ventral neuron doublet VD10 and DD5 over which the DTC would normally change migration from ventral to dorsal.

5.5 DAF-12 temporal regulation

DAF-12 is one of many temporal regulators of developmental events in *C. elegans*, also referred to as heterochronic genes. DAF-12 is mainly responsible for regulating the dauer stage that the worms can enter under unfavourable conditions (Ambros, V. 1997). Previous work has shown the involvement of DAF-12 in regulating the expression of *unc-5* for the second migration phase of the DTC. The hypomorphic allele *daf-12(rh61)* is a mutation in the DBD of the protein. It also does not display upregulation of *unc-5* during the second migration phase. However, the null mutant allele *daf-12(rh61rh411)* which is a mutation both in the DBD and the LBD, displays *unc-5* expression during the second migration phase (Ambros, V. 1997). It is not yet known why these different alleles show different *unc-5* expression patterns but this suggested that there might be other elements acting in parallel to DAF-12 in the regulation of *unc-5* expression. For this reason we decided to investigate whether EGL-20/WNT and EGL-17/FGF spatial cues were involved with DAF-12 in the regulation of *unc-5* during the second migration phase.

In order to understand how *daf-12* might have a role in the guidance of the DTC along its migration path, we obtained mutants of *daf-12* to observe the phenotypes that arise when combined with *egl-20* and *egl-17*. The *daf-12(rh411)* is a null allele of *daf-12*. It contains two premature, in frame, stop codons within the ligand binding domain (LBD) and DNA binding domain (DBD) (Ambros, V. 1997).

Double mutants of *egl-17; daf-12(rh411)* did not show any significant increase in DTC migration defects (see Table 2) and were very similar to *unc-5GFP* wild-type worms. When *daf-12(rh411)* was put into an *egl-20* mutant phenotype we observed a

similar frequency of third phase longitudinal DTC migration defects (16%, N=110) to that observed in the *egl-20* single mutants (12%, N=56). None of the double mutants of *egl-20; daf-12* or *egl-17; daf-12* displayed any significant differences from the single mutants of *egl-17* and *egl-20* even when the *daf-12* mutation was introduced.

In triple mutants of *egl-20; egl-17 daf-12(rh411)*, we observed a non significant decrease in the frequency of third phase longitudinal migration defects (down to 9%) and a significant increase in the frequency of first phase migration defects (32%, N=112) compared to *egl-20; egl-17*. These first migration defects were composed mostly of late migrations along the ventral A-P axis of the worm. This could indicate that there is an interaction with DAF-12, but it is difficult to interpret without *daf-12; unc-5GFP*. Also, *unc-5* expression was still present in the triple mutants indicating that EGL-20/WNT and EGL-17/FGF are not involved with DAF-12 in the regulation of *unc-5* expression. Up-regulation of *unc-5* during the second migration phase is most likely a temporally regulated event rather than a spatially regulated one.

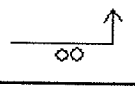
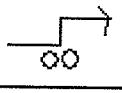
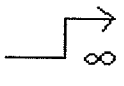
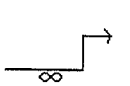
			1st migration phase		3rd migration phase		1st &3rd mig phase	
								
Strains/phenotypes	N	wild-type	precocious	late	zig-zag	longitudinal	early+long.	late+long.
egl-17;daf-12(rh411);unc-5GFP	92	98.91%	0.00%	1.09%	0.00%	0.00%	0.00%	0.00%
egl-20;daf-12(rh411);unc-5GFP	110	80.73%	0.92%	1.83%	0.00%	15.60%	0.92%	0.00%
egl-20;egl-17;daf-12(rh411);unc-5GFP	112	59.93%	0.00%	32.14%	0.00%	8.04%	0.00%	0.89%

Table 3. Percentages of the phenotypes scored for *daf-12* mutants.
All worms were scored under the compound microscope. Worms were picked onto an agarose pad (see Materials and Methods) and immobilized using 10% sodium azide solution in the agarose pad. The table represents the percentages of the total N number of worms belonging to each phenotype (as indicated at the top of the table) for each *C. elegans* strain.

5.6 *unc-5(ev585)* mutation in an *egl-20; egl-17* mutant background

We tested to see if in an *unc-5* mutant background *egl-20* and *egl-17* would have the same effect on DTC migration as the *egl-20;egl-17* double mutant. The triple mutants of *unc-5(ev585) egl-20; egl-17* displayed many migration defects. These defects were characterized by 23% of first phase late migration defects along with 17% third phase longitudinal migration defects. We also expected to see some migration defects affecting the second migration phase since the expression of *unc-5* is essential for the migration of the DTC from ventral to dorsal. This phenotype was observed at a frequency of 41%. This data is however inconclusive since we did not have the chance to observe the single mutant of *unc-5(ev585)* which would have provided some insight into what the interactions between UNC-5, EGL-20 and EGL-17 might be. Mutants of *unc-5* only present second migration phase defects. Therefore precocious migrations are reduced when the function *unc-5* is impaired or when DAF-12 is eliminated. The second migration phase requires DAF-12 for *unc-5* up-regulation.

			First phase		Third phase		Second phase
Strains/ Phenotypes	N	wild-type	precocious	late	zigzag	longitudinal	ventro-dorsal
unc-5GFP (wild-type)	114	93.0%	7.0%	0.0%	0.0%	0.0%	0.0%
egl-20;egl-17;unc-5GFP	64	59.0%	20.3%	3.1%	3.1%	14.1%	0.0%
unc-5(ev585) egl-20; egl-17;unc-5GFP	86	15.1%	3.5%	23.3%	0.0%	17.4%	40.7%

Table 4. Percentage of phenotypes for *unc-5GFP(ev585); egl-20; egl-17 mutant worms.*
All worms were scored under the compound microscope. Worms were picked onto an agarose pad (as described in Section 4.5.1) and immobilized using 10% sodium azide solution. The table represents the percentages of the total N number of worms belonging to each phenotype (as indicated at the top of the table) for each *C. elegans* strain.

5.7 Investigation of Downstream Effectors of WNT and FGF Signaling Pathways

EGL-20/WNT and EGL-17/FGF are part of two very important and intricate signaling pathways. Based on the observations made thus far, it is reasonable to think that they are both involved in the proper migration of the DTC and that without them the DTC lacks the proper positional cues to correctly complete its migration. We decided to further investigate EGL-20/WNT and EGL-17/FGF pathways to pinpoint which components are involved in directing the DTC along its three migration phases. To do so, we referred to both wormbook.org as well as wormbase.org for genes involved in the WNT and FGF signaling pathway of *C. elegans*. We compiled a list of the genes from both signaling pathways which are expressed in the DTC or the somatic gonad of *C. elegans* hermaphrodite (Table 4, Figure 5). The goal was to reproduce the phenotype observed in *egl-20; egl-17* double mutant by combining *egl-20* or *egl-17* mutants with mutant components of the EGL-17/FGF or EGL-20/WNT signaling pathways respectively. Observing the different double mutants combinations would help us to identify downstream elements involved in regulating DTC migration.

One problem encountered was that some of mutants are lethal and could not be examined in late larval stages. To bypass this problem we resorted to RNA interference (RNAi) to knock down the genes of interest. A few different RNAi strains for genes such as *egl-15* and negative control pPD129.36 were transformed into HT115 bacterial cultures. This *E. coli* strain is capable of producing RNAi strands in large amounts. The bacterial cultures are then spread out on the appropriate carbenicillin and IPTG plates (see Materials and Methods section 4.11) and left to dry to create a lawn of *E. coli*. Two young (L1-L2 stage) *C.elegans* hermaphrodites were picked onto each plate and left to

grow and reproduce at 20°C. Their progeny were scored under the compound microscope for DTC migration defects as outlined above.

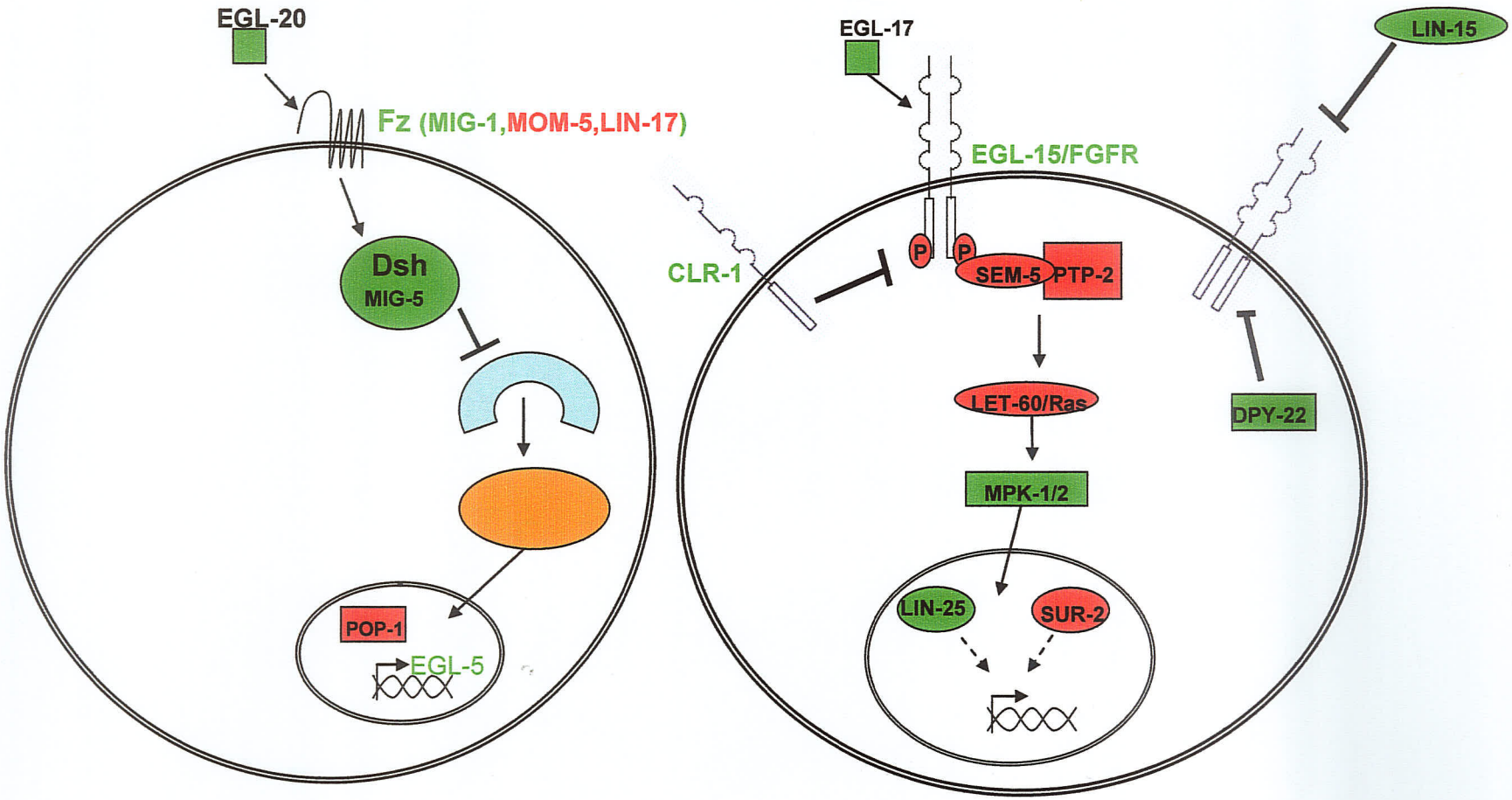


Figure 8. EGL-20/WNT and EGL-17/FGF signaling pathways.
This figure represents the components for both signaling pathways that are expressed in the somatic gonad or the DTC. Components indicated in green were successfully tested while components in red were not tested due to time restraints or lethal phenotypes of the mutant strains.

EGL-17/FGF components	Function
egl-15	EGL-17 receptor
clr-1	Receptor tyrosine phosphatase
dpy-22	Adaptor protein involved in WNT+ Ras pathway
lin-25	Nuclear protein downstream of let-60
ptp-2	Protein tyrosine phosphatase
mpk-1	Mitogen-activated protein (MAP) kinase
let-60	Ras relate GFPase
sur-2	Mediator protein downstream element of let-60-ras mediated signaling
sem-5	Downstream effector (interacts with egl-15)
lin-15	Involved in vulva development
EGL-20/WNT components	
mom-5	EGL-20 receptor (frizzled homologue)
mig-1	EGL-20 receptor (frizzled homologue)
lin-17	EGL-20 receptor (frizzled homologue)
egl-5	Down stream gene regulated by WNT
pop-1	Transcription factor
mig-5	Disheveled homologue

Table 5. Investigation of Downstream Effectors of the WNT and FGF Signaling Pathways.
The table represents all the downstream elements of EGL-20/WNT and EGL-17/FGF that are expressed in the DTC and that we suspect might have a role in guiding the DTC along its migration. These genes are targeted using crosses with mutant worms or by feeding RNAi to the worms in order to get knockdown.

Strains	N	DTC migration phenotype	
		% wild type	% mutant
unc-5-GFP	114	93	7
egl-17;unc-5GFP	49	94	6
egl-20;unc-5GFP	56	88	12
egl-20;egl-17;unc-5GFP	64	59	41
EGL-17/FGF components			
egl-15(n484);unc-5GFP[1]	105	80	20
egl-15(n484);egl-20;unc-5GFP[1]	94	84	16
clr-1;unc-5GFP[2]	92	72	28
clr-1;egl-20;unc-5GFP[2]	161	63	37
dpy-22;unc-5GFP	115	45	55
dpy-22;egl-20;unc-5GFP	106	62	38
unc-32;unc-5GFP	94	64	36
mpk-1unc-32;unc-5GFP	117	56	44
mpk-1unc-32;egl-20;unc-5GFP	124	56	44
lin-25;unc-5GFP	90	84	16
lin-25;egl-20;unc-5GFP	114	49	51
lin-15;unc-5GFP	97	64	36
lin-15;egl-20;unc-5GFP	146	27	73
lin-15;egl-20;egl-17;unc-5GFP	108	20	80
EGL-20/WNT components			
mig-1;unc-5GFP	95	84	16
mig-1;egl-17;unc-5GFP	103	62	38
mig-5;unc-5GFP	93	77	23
mig-5;egl-17;unc-5GFP	96	60	40
egl-5;unc-5GFP	93	91	9
egl-5;egl-17;unc-5GFP	118	43	57

Table 6. Scoring results for components of EGL-17/FGF and EGL-20/WNT signaling pathways.

Compilation of all strains obtained for the investigation of the downstream effectors of EGL-20/WNT and EGL-17/FGF. N number of worms were counted for each strain and each DTC migration was either categorized as wild-type or as mutant. Mutants are any phenotype that diverges from the wild-type phenotype where the DTC turns over the doublet VD10 and DD5. This includes both the first and third migration defects. All counts were done at 20°C unless indicated otherwise and scored as indicated in materials and methods.

[1]Counts were done at 25°C. [2]Counts were done at 16°C since *clr-1* is a temperature sensitive strain.

5.7.1 EGL-17/FGF components

5.7.1.1 *egl-15(n454)* allele did not display a significant change in DTC migration defects.

EGL-15/FGFR encodes the only FGF receptor in the *C. elegans* genome. To test the involvement of EGL-15/FGFR, we first used *egl-15(n484)* allele crossed with *egl-20* to try to reproduce the *egl-20; egl-17* phenotype. This double mutant did not display any statistically significant increase or decrease in the number of DTC migration defects compared to wild-type animals (Table 6). This allele is a hypomorphic mutation of *egl-15* which means that it is a partial knockdown of gene function. Indeed, *egl-15(n484)* only eliminates isoforms 5A (5B is still remaining) (DeVore, D.L. *et al.*, 1995). It is necessary to use such a mutant since EGL-15/FGFR is essential to the survival of *C. elegans*. Therefore, the weak nature of this allele is most probably responsible for the lack of a noticeable phenotype.

Our second approach was to use the temperature sensitive (ts) *egl-15(n1477)X* allele which corresponds to a nonsense mutation in the C-terminal domain (CTD) of the receptor. The mutation of the 3'end of the coding region is reported to be required for sex myoblast (SM) chemoattraction (Goodman, S.J., 2003). Both isoforms 5A and 5B are reduced in this mutant but are not eliminated (DeVore, D.L. *et al.*, 1995). This allele was also crossed into an *egl-20* mutant background to obtain double mutants of *egl-20; egl-15(n1477)* and see if we can obtain a significant phenotype. To date, we have not been able to create this double mutant but we believe that it would be a good indicator of the level of involvement of this receptor in mediating information for the migration of the posterior DTC.

The third approach that we took in order to see if EGL-15/FGFR is involved in DTC migration was to use RNAi. The experiment was repeated three times in order to confirm the phenotype observed using pPD129.39 as a control and *egl-15(X5E24)*, both transformed into HT115 cells as RNAi strains. The first experiment did not give any observable phenotype however we decided to reproduce the experiment because we suspected that the RNAi feeding plates might have been too old and could have affected the results. After repeating the experiment a second time, we did not observe any significant difference between the different strains and we are not confident that the RNAi is effective.

5.7.1.2. *clr-1*, a negative regulator of EGL-15/FGFR, displays an increase in first phase precocious migrations defects in an *egl-20* mutant background.

CLR-1 is a negative regulator of EGL-15/FGFR, which is the only FGF receptor for EGL-17/FGF in *C. elegans*. Under normal circumstances, CLR-1 suppresses signaling through EGL-15/FGFR. Without CLR-1, EGL-15/FGFR signaling is increased (DeVore, D.L. *et al.*, 1995).

In *clr-1* single mutants we observed a number of first phase late migration defects (24%, N=92) (Table 6). These were characterized by ventral to dorsal migrations at a more posterior position along the A-P axis of the worm. When crossed to an *egl-20* mutant background, *clr-1; egl-20* double mutants were observed to have a shift in their DTC migration defects, compared to *clr-1* single mutants. Overall, we were successful in recreating the *egl-20; egl-17* double mutant phenotype in the *clr-1; egl-20* double mutants (Table 5). The first phase late migration defects observed in *clr-1* single mutants were for the most part shifted to first phase precocious migration defects (12%, N=161) in *clr-1; egl-20* double mutants. These were also accompanied by third phase

longitudinal migration defects (19%, N=161). The increase sensitivity of EGL-15/FGFR for EGL-17/FGF signaling does not seem to be able to compensate for the lack of EGL-20/WNT in double mutants of *clr-1; egl-20*. This is particularly true for the first migration phase (discussed in section 6.3.1). Also, the increase in third phase longitudinal migration defects (approximately 19%) in *clr-1; egl-20* mutants are a characteristic phenotype of *egl-20* single mutants.

5.7.1.3. Dpy-22 phenotype is inconclusive due to the short nature of the worm.

DPY-22 is a downstream element of EGL-17/FGF signaling pathway and is reported to be a negative regulator or the RTK/Ras signaling pathway (Aroian, R.V. *et al.*, 1990). Dumpy (Dpy) worms are easily identified under the dissecting microscope due to their short body length. Single mutants of *dpy-22(sy622)X* mutants displayed a large proportion of first phase late migration defects (53%, N=115) (Table 6). This was also observed in the double mutant of *egl-20; dpy-22(sy622)X* worms at a slightly lower frequency (36%, N=106). We suspect that the lack of difference in phenotypes between *dpy-22* single mutants and *egl-20; dpy-22* double mutants and the first phase late migration phenotype might be due to the stumpy nature of the Dpy worms. Cell targeted RNAi might be a better approach to test this gene in its involvement in DTC migration.

5.7.1.4. Single and double mutants of *mpk-1* do not display differences in DTC migration defects.

MPK-1 is part of the downstream elements of EGL-17/FGF signaling pathway and is expressed in the somatic gonad. The single mutants of *mpk-1* were accompanied by the *unc-32* mutation in order to be able to identify worms carrying the *mpk-1* mutation. The uncoordinated (Unc) phenotype is easily recognized inability of the worm to promptly move forward or backwards when tapped on the head or the tail respectively (Brenner, S.,

1974). The *unc-32(e189)III* in particular tends to coil up on itself move very little on the agarose plate. Single mutants of *unc-32* and double mutants of *mpk-1(kul); unc-32(e189)III* displayed very similar phenotypes with mostly first phase late migration defects (34%, N=95 and 42%, N=121 respectively) compared to the wild-type phenotype. Triple mutants of *mpk-1(kul); unc-32(e189); egl-20* did not display a significantly different phenotype from the single and double mutants mentioned above. It also displayed first phase late migration defects (43%, N=124) (Table 6).

The lack of significant changes in the DTC migration defects between the different *mpk-1* mutants suggests that the mutation is either not significant or another factor is masking the true involvement of MPK-1 in the posterior DTC migration. Indeed, *mpk-1(kul)* allele is a hypomorphic allele which means that it is a partial loss of function (Sundaram, M.V. *et al.*, 1996). Another approach such as RNAi knockdown might be more efficient in observing the appropriate phenotype.

5.7.1.5. *lin-25* mutants reinforce EGL-17/FGF's specific role in the first migration phase.

LIN-25 is a downstream nuclear protein of the RTK/Ras signaling pathway. It might act with a mediator complex member SUR-2 in assembly of the transcription machinery or as a transcription factor itself. *lin-25(n545)* is a hypomorphic allele of this gene at 20°C (Tuck, S. and Greenwald, I., 1995).

Single mutants of *lin-25(n545)V* did display some migration defects mostly characterized by first phase precocious migration defects (16%, N=90)(Table 6). We were able to reproduce the double mutant phenotype of *egl-20; egl-17* in the case of *lin-25; egl-20* double mutants. The double mutant strain *lin-25(n545); egl-20* did exhibit the same first phase precocious migration defects (21%, N=114) as the single mutant but also

displayed third phase longitudinal migration defects (30%). Single and double mutants of *lin-25* mutants seem to underline EGL-17/FGF's role in the first migration as well as the role of EGL-20/WNT in the third migration phase.

5.7.1.6. LIN-15

LIN-15 is a negative regulator of LET-23/EGFR. Mutants of *lin-15* display what is called a multivulva (Muv) phenotype (Huang, L.S. *et al.,* 1994). This means that more than one vulva is observed along the ventral side of *C.elegans* hermaphrodite; however most of the extra vulvas are non-functional. Since EGL-17/FGF is normally expressed from the vulvul precursor cells (VPC) these multiple vulvas can potentially all express EGL-17/FGF. We first characterized the expression of EGL-17/FGF using *egl-17* gene tagged with a GFP reporter construct in order to observe the expression of *egl-17* throughout worm development. Expression of *egl-17* starts very early on when the VPCs are lined up on the ventral side of the animal and persists into late vulva development in the adult hermaphrodite (Figure 9A-E). Also, in *lin-15*; *egl-17GFP* single mutant worms, which are Muv, have 'extra' vulvas both anterior and posterior to the 'original' vulva. The number of extra vulvas anterior to the original vulva varied between 1 and 2 whereas an average of only one 'extra' vulva was noticed posterior to the 'original' vulva. Most of the anterior 'extra' vulvas displayed expression of *egl-17GFP* (84%) in contrast to only 21% of the posterior 'extra' vulvas displayed *egl-17GFP* expression (Figure 10). Regarding the DTC migration phenotype, in single mutants of *lin-15; unc-5GFP* we observed first phase late migration defects (37%, N=105). This could indicate that the excess EGL-17/FGF signaling from the 'extra' vulvas might be forcing the DTC to migrate farther along the A-P axis before *unc-5* up-regulation.

Double mutants of *lin-15; egl-20* displayed mostly third phase longitudinal migration defects (52%, N=146) where the DTC fails to reflex back to the mid-body of the animal. This is a characteristic detected of the *egl-20* single mutant and could suggest that EGL-20/WNT acts to repulse the DTC during the third migration phase. These mutants also displayed fewer first phase late migration defects (14%) than *lin-15* single mutants (37%) (Table 6).

Triple mutants of *egl-20; egl-17; lin-15* displayed first phase late migration defects and third phase longitudinal migration defects at a frequency of approximately 28% and 48% respectively (N=108). This could indicate that the migration defects observed in the single and double mutants of *lin-15* and *lin-15; egl-20* respectively are not dependent on the excess of EGL-17/FGF being expressed in the 'extra' vulvas.

Although LIN-15 is not a direct suppressor of EGL-15/FGFR, it could be part of the same additional regulation mechanism independent of EGL-20/WNT and EGL-17/FGF. Since it is expressed from the hypodermis surrounding the vulva it is possible that LIN-15 affects other components that are directly related to the migration of the DTC.

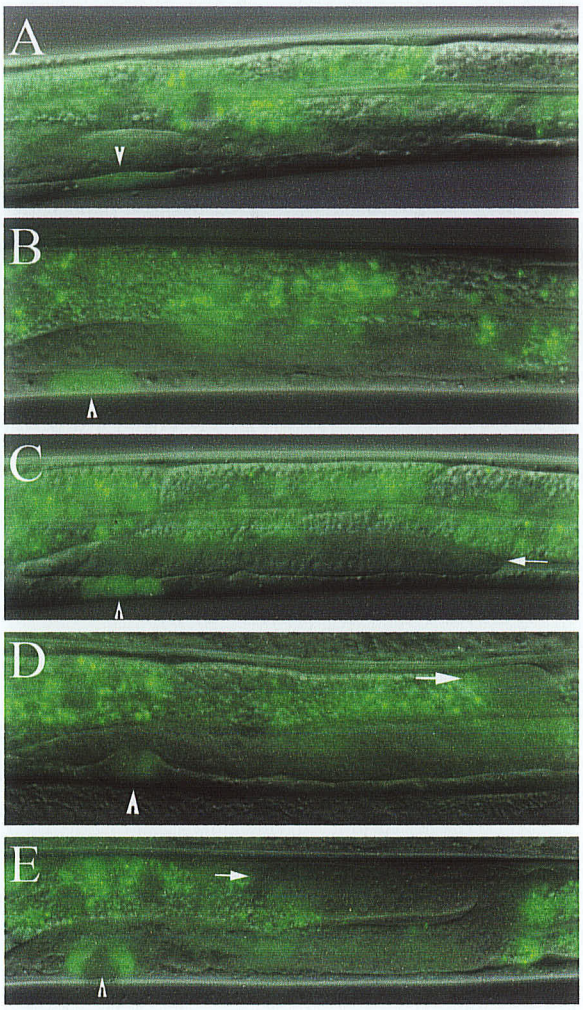


Figure 9. *egl-17GFP* expression in the developing vulva of hermaphrodite C.elegans.
DIC and fluorescent picture overlays of *C.elegans* vulva and posterior gonad arm. The five different panels represent different stages (starting at **A** through **E**) of vulva development along with *egl-17GFP* expression pattern. *egl-17GFP* is expressed very early in the developing vulva and its expression persists late into the adult stage of *C.elegans* development. Arrowheads indicate the position of the developing vulva (which expresses *egl-17GFP*) and the arrow shows the DTC along its migration pattern.

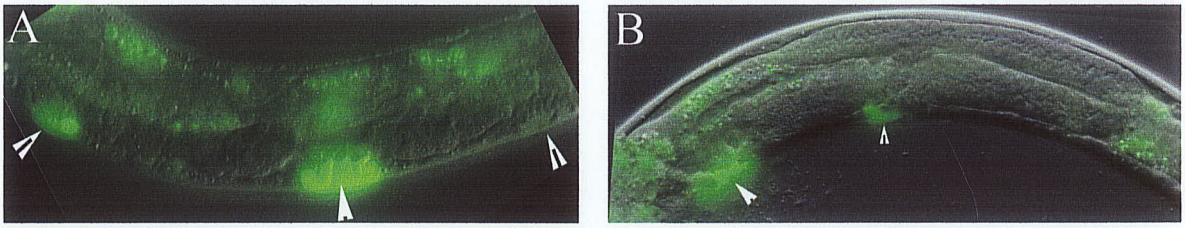


Figure 10. DIC and fluorescent overlay of *lin-15; egl-17GFP* worms.
These worms display **mu**ltiple vulvas (Muv) indicated by the arrowheads. Full arrowhead indicates the position of the "original" vulva and smaller hollow arrowheads depict the anterior and posterior "extra" vulvas. **A**- depicts a worm with 2 "extra" vulvas, one anterior and one posterior to the "original" vulva. The "original" vulvas as well as the anterior "extra" vulva display *egl-17GFP* expression whereas the posterior vulva does not. **B**- shows one of the few cases where the "extra" posterior vulva did display *egl-17GFP* expression along with the "original" vulva.

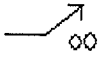
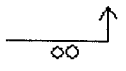
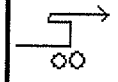
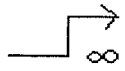
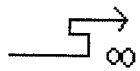
Strains/ Phenotypes	N	 wild-type	 precocious	 late	 zigzag	 longitudinal	 precocious+ longitudinal	 precocious + Zigzag
egl-15(n484);unc-5GFP	105	80.00%	7.62%	0.00%	0.00%	0.95%	11.43%	0.00%
egl-15(n484);egl-20;unc-5GFP	94	84.04%	6.38%	0.00%	0.00%	4.26%	6.38%	0.00%
clr-1;unc-5GFP	92	72.83%	2.17%	25.00%	0.00%	0.00%	0.00%	0.0%
clr-1;egl-20;unc-5GFP*	**161**	**64.60%**	**12.42%**	**4.35%**	**4.97%**	**6.83%**	**5.59%**	**1.24%**
dpy-22;unc-5GFP	115	44.83%	0.86%	53.45%	0.00%	0.00%	0.00%	0.86% (V-D defect)
dpy-22;egl-20;unc-5GFP	**106**	**61.32%**	**1.98%**	**35.85%**	**0.94%**	**0.00%**	**0.00%**	**0.00%**
unc-32;unc-5GFP	95	63.16%	3.16%	33.68%	0.00%	0.00%	0.00%	0.00%
mpk-1unc-32;unc-5GFP	121	57.85%	0.00%	42.15%	0.00%	0.00%	0.00%	0.00%
mpk-1unc-32;egl-20;unc-5GFP	124	56.45%	0.00%	43.55%	0.00%	0.00%	0.00%	0.00%
lin-25;unc-5GFP	90	80.00%	15.56%	3.33%	0.00%	1.11%	0.00%	0.00%
lin-25;egl-20;unc-5GFP	**114**	**46.49%**	**21.05%**	**2.63%**	**8.77%**	**8.77%**	**9.65%**	**2.63%**
lin-15;unc-5GFP	105	60.95%	0.00%	37.14%	0.00%	0.95%	0.95%	0.00%
lin-15;egl-20;unc-5GFP	**146**	**26.03%**	**8.22%**	**13.70%**	**0.00%**	**37.67%**	**8.90%**	**5.48%(late+long)**
lin-15;egl-20;egl-17;unc-5GFP	**108**	**20.37%**	**4.63%**	**27.78%**	**0.00%**	**37.96%**	**4.63%**	**4.63%(late+long)**

Table 7. EGL-17/FGF components.
This table summarizes the percentages or each phenotype (as indicated at the top of the table) observed of N total worms for each mutant strain. Data in bold indicates statistical significance. Statistical significance indicates that the data is divergent from the *unc-5* wild-type. Asterisk indicates that this strain was counted at 16°C as it is temperature sensitive.

5.7.2. EGL-20/WNT Components

5.7.2.1. *mig-1/Frizzled* receptor mutation mimics *egl-20; egl-17*double mutant phenotype in an *egl-17*mutant background

MIG-1 encodes a receptor protein homologous to the Frizzled receptor in Drosophila and is one of four WNT receptors in *C. elegans* (Eisenmann, D., 2005). Single mutants of *mig-1* were scored for migration defects and showed no statistically significant difference from *unc-5GFP* wild-type worms (Table 7). This came as a surprise since we anticipated the single mutant of *mig-1* to have third phase longitudinal migration defects as observed in single mutants of *egl-20*. However, since MIG-1 is one of four frizzled receptors for the WNT signaling pathway it might not be so surprising that we did not observe any significant phenotype due to functional redundancy.

When put into an *egl-17* mutant background, the *mig-1; egl-17* double mutants displayed many DTC migration defects similar to the double mutants *egl-20; egl-17*. In fact, *egl-20; egl-17* and *mig-1; egl-17* are not significantly different from one another indicating that we were successful in reproducing the mutant phenotype. The migration defects were characterized by first phase precocious migration defects (17%, N=103) and third phase longitudinal migration defects (18%).

5.7.2.2. *mig-5/Dishevelled* mutants also display an increase of first and third phase DTC migration defects.

MIG-5 is homologous to the Disheveled WNT protein in Drosophila and acts downstream of MIG-1/WNT receptor (Eisenmann D., 2005). The *mig-5(rh147)* allele was crossed out of the genetic balancer, *mlnI(dpy-10(e128))mls14 II* which is an inversion (Walston, T. *et al.,* 2006). These worms had a Dpy phenotype, mentioned previously, giving the worms a short stature.

Single mutants of *mig-5(rh147)* did display some DTC migration, mainly exhibiting first phase late migration defects at a frequency of 12% and third phase longitudinal migration defects at 16 % (N=106) (Table 7).

Double mutants of *mig-5(rh147)* also displayed third phase longitudinal migration defects at a frequency of approximately 16%(N=101). However these were accompanied by first phase precocious DTC migration defects (21%) instead of first phase late migrations as seen in the single mutants. This double mutant phenotype mimics *egl-20; egl-17* DTC migration defects. Since the third phase longitudinal migration defect does not seem to be affected by the presence or absence of the *egl-17* mutation, this suggests that the third migration phase might be more dependent on the EGL-20/WNT signaling pathway. As for the first migration phase we noticed a shift from late migrations in the single mutants to precocious migrations in the double mutants. This could indicate that both EGL-17/FGF and EGL-20/WNT must have a role in regulating the first migration phase.

5.7.2.3. *egl-5* mutation in an *egl-17*mutant background reveals further involvement of the WNTs in DTC migration

EGL-5 is a transcription factor which is part of the downstream elements of the WNT signaling pathway (Ferreira, H. *et al.*, 1999). This Hox gene is expressed in the somatic gonad as well as the DTC (Eisenmann, D.M., 2005). *egl-5(n945)III* allele was easily characterized due to the coiler phenotype. Single mutants of *egl-5(n945)III* do not display any significant DTC migration defects and do not differ significantly from the wild-type *unc-5GFP* (Table x).

Double mutants of *egl-5; egl-17* did display a significant increase in DTC migration defects, mimicking the severity of *egl-20; egl-17*. These double mutants displayed 46%

(N=118) first phase precocious migration defects along with 9% third phase longitudinal migration defects. The frequency of first phase migration defects is unlike that found in *egl-20; egl-17* double mutants (20%). In this instance there is a much higher (46%) incidence of first phase precocious migration defects. Also, the frequency of third phase migration defects is much lower in the *egl-5; egl-17* double mutants (9%) compared to *egl-20; egl-17* double mutants (20%) (Table 7). This suggests that *egl-5* has a larger involvement in the first migration phase compared to the third migration phase. This is in contrast to *mig-1* and *mig-5* which have both been shown to have an active involvement in the first and third migration phase of the DTC. Also, since *egl-5; egl-17* mutants do not display many third phase migration defects this could indicate that something else is responsible for the third migration phase (since third phase migration defects were observed in *mig-1*and *mig-5* mutants).

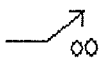
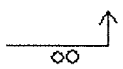
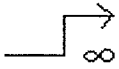
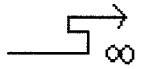
EGL-20/Wnt components			First mig. defects		Third mig. defects		Combination of 1st and 3rd	
								
Strains/ Phenotypes	N	wild-type	precocious	late	zigzag	longitudinal	precocious+ longitudinal	precocious + zigzag
mig-1;unc-5GFP	95	84.21%	7.37%	8.42%	0.00%	0.00%	0.00%	0.00%
mig-1;egl-17;unc-5GFP	**103**	**61.17%**	**16.50%**	**2.91%**	**12.62%**	**3.88%**	**2.91%**	**0.00%**
mig-5;unc-5GFP	**106**	**67.92%**	**3.77%**	**12.26%**	**8.49%**	**6.60%**	**0.94%**	**0.00%**
mig-5;egl-17;unc-5GFP	**101**	**57.43%**	**20.79%**	**4.95%**	**0.99%**	**5.94%**	**9.90%**	**0.00%**
egl-5;unc-5GFP	93	91.40%	5.38%	1.08%	2.15%	0.00%	0.00%	0.00%
egl-5;egl-17;unc-5GFP	**118**	**44.07%**	**45.76%**	**0.85%**	**0.85%**	**2.54%**	**4.24%**	**1.69%**

Table 8. EGL-20/WNT components.
This table summarizes the percentages or each phenotype (as indicated at the top of the table) observed of N total worms for each mutant strain. Data in bold indicates statistically significance. Statistical significance indicates that the data is divergent from the *unc-5* wild-type.

6. DISCUSSION

6.1 *egl-20; egl-17* double mutant phenotype

The distal tip cell must go through three migration phases to properly guide the developing gonad arms of *C. elegans* hermaphrodites. The first and third migration phases occur along the A-P axis on the ventral and dorsal side or the animal, respectively, while the second migration phase occurs along the V-D axis (Headgecock *et al.*, 1987). The DTC depends on multiple temporal and spatial cues to guide it along its three migration phases. It can decide to respond or not to the variety of cues it encounters and the response to a specific cue can also vary. This is dependent on a number of factors such as spatial position of the DTC, the receptors it presents, other surrounding signaling cues, developmental timing and much more. The observations made in the double mutants of *egl-20; egl-17* clearly demonstrates that there is a change in the rate of migration along the ventral side of *C. elegans* hermaphrodites during the first migration phase of the DTC. However, we were unable to determine if this change in rate meant that the DTC was moving at a slower rate or if the DTC was initiating the second migration phase early in development strictly based on scoring *egl-20; egl-17* double mutant worms. We further investigated EGL-17/FGF and EGL-20/WNT's control on *unc-5* expression along with comparing the gonad development to vulva development.

Initially we hypothesized that the posterior DTC of double mutants of *egl-20; egl-17* was initiating the second migration phase at an earlier developmental time than wild-type counterparts. This would of meant that *unc-5* expression was up-regulated in the posterior DTC earlier in development driving the DTC from ventral to dorsal at a more

anterior position along the A-P axis. However, further experiments proved our hypothesis to be wrong.

First, the double mutants *egl-20; egl-17* clearly displayed non synchronized migration of the anterior and posterior DTCs. In these mutants the anterior arm was migrating at the appropriate rate but the posterior DTC lagged behind, unlike wild-type DTCs which migrate in a highly synchronized manner. This was the first indication that the posterior DTC was moving at a slower rate.

Secondly, we observed that *unc-5Lac-Z* was being expressed regardless of the position of the posterior DTC along the A-P axis in *egl-20; egl-17* double mutant worms. Had the posterior DTC been initiating the second migration phase early in development (in double mutants of *egl-20; egl-17)* we would have predicted that the posterior DTC to express β-gal earlier than the anterior DTC. This would have suggested that EGL-20/WNT and EGL-17/FGF were both involved in the regulation of *unc-5* expression in the posterior DTC. However, this is not what we observed. Instead we observed that the developmental timing of *unc-5* expression was not altered in these mutants. This hypothesis was supported by observing β-gal staining in both the anterior and posterior DTCs. We also noticed that the β-gal staining was initiated regardless of the position of the posterior DTC which could suggest that *unc-5* expression is regulated by a temporal factor instead of a spatial factor. Therefore, regardless of the position of the anterior or posterior DTC *unc-5* is expressed with the appropriate developmental timing.

These data suggest that EGL-20/WNT and EGL-17/FGF do not regulate the transcription of *unc-5* since the timing of the *unc-5Lac-Z* expression was not altered in either the anterior or posterior DTC of *egl-20; egl-17* mutant worms. We propose that

the positioning of the posterior DTC is dependent on spatial cues whereas *unc-5* up-regulation is likely dependent on temporal cues.

Given these data, we rejected the hypothesis that the posterior DTC in *egl-20; egl-17* was initiating the second migration phase at an earlier developmental time and instead accepted the alternative hypothesis; that it might be moving at a slower rate or is initiating the first migration at a later developmental time. Our observations also indicated that both the EGL-20/WNT and EGL-17/FGF have overlapping roles in providing spatial A-P guidance to the posterior DTC and have no effect on regulating *unc-5* transcription. Cell migration for EGL-20/WNT and EGL-17/FGF pathways is not unique to the DTC since both are involved in guidance of Q neuroblast and sex myoblast (SM), respectively, along the A-P axis of *C. elegans*. Their gradients and specific area of expression are important in giving the appropriate spatial cues to a variety of migrating cells during development (Eisenmann, D.M., 2005; DeVore, D.L. *et al.*, 1995).

6.1.1 Building a model for the third migration phase

In order to put this information into perspective we constructed a model of the third migration phase based on the observed longitudinal migration defects of *egl-20* and *egl-20; egl-17* mutants. Assuming that EGL-20/WNT and EGL-17/FGF are providing spatial information to the DTC, we hypothesized that EGL-20/WNT (being expressed form the tail region) is supplying a repulsion effect while EGL-17/FGF (expressed by the VPCs in the vulva) supplies an attraction effect on the posterior DTC. The combination of push and pull by both protein gradients helps the DTC to migrate back into the mid-body along the dorsal side and prevents third stage longitudinal migration defects. The overlapping

roles that EGL-20/WNT and EGL-17/FGF play in guiding the DTC are unveiled when we look at double mutants *egl-20; egl-17*. In the single mutants of *egl-20,* we observed some third phase longitudinal migration defects (12%, N=56) which indicates that the push that is supplied by EGL-20/WNT is necessary for the DTC to return to the dorsal mid-body (Table 8). Interestingly the frequency of third phase longitudinal migration defects does not change significantly in *egl-20; egl-17* double mutants compared to *egl-20* single mutants. Instead we noticed that these third phase migration defects had switched from a 'zigzag' phenotype to a straight longitudinal phenotype. In the 'zigzag' phenotype the DTC initiates its migration back to the mid-body but then aberrantly migrates to the tail. Contrarily, in *egl-20; egl-17* double mutants the DTC does not hesitate and migrates straight toward the tail. This obervation strongly suggests that the higher concentration of EGL-20/WNT in the tail is necessary to push the DTC back to the mid-body but is not sufficient. Indeed, it seems that EGL-17/FGF also has a role in guiding the DTC in its third migration phase along with EGL-20/WNT. This last statement is supported by the fact that there is a considerable decrease in the hesitation of the DTC during the third migration phase of *egl-20; egl-17* double mutants.

	1st mig. phase	3rd mig. phase
EGL-20	?	repulsion
EGL-17	?	attraction

Table 9. Third migration phase model.

6.1.2 Building a model for the first migration phase

In order to complete our model concerning the response of the DTC during the first migration phase we compared the developmental stages of gonad development in relation to vulva development. When comparing the posterior DTCs at the same migration phase of a wild-type and an *egl-20; egl-17* mutant worm, we noticed that the mutant vulva was much more developed than the wild-type vulva. This indicated that the posterior DTC was moving at a slower rate along the ventral side in *egl-20; egl-17* double mutants. We were then able to develop our model of what might be happening in the first migration phase regarding EGL-20/WNT and EGL-17/FGF working as spatial cues. Contrary to the third migration phase, EGL-17/FGF seems to have a repulsion effect on the DTC while EGL-20/WNT attracts it along the ventral side during the first migration phase.

	1st mig. phase	3rd mig. phase
EGL-20	attraction	repulsion
EGL-17	repulsion	attraction

Table 10. First migration phase model.

This model helps outline the opposing but overlapping roles of EGL-20/WNT and EGL-17/FGF in the first and third migration phase of the DTC but also underlines another important element. The DTC changes its response towards EGL-20/WNT and EGL-17/FGF between the first and third migration phases. This is very important since the change in response contributes to the proper migration of the posterior DTC. Both EGL-20/WNT and EGL-17/FGF have been reported to have both and attractive and repulsive role in regulating the migration of different cells (Whangbo J. and Kenyon C., 1999; Fleming, T.C. *et al.*, 2005). A number of different mechanisms can modify the response of a cell to a signaling. Changes in levels of cyclic nucleotides can act as a second messenger to interpret signals encountered by the cell (Livesey, F.J., 1999).

Perhaps the change in distance of the DTC from both sources of EGL-20/WNT and EGL-17/FGF has an important impact on its response of the DTC to these cues. Also, up-regulation and down regulation of genes at key stages during cell migration may contribute to the pathfinding of a migrating cell. During the second migration phase, the DTC changes its ECM substrate from the ventral muscle band to the dorsal muscle band. *C. elegans* integrin homologues , *ina-1* and *pat-2*, have been shown to be essential in stopping the DTC migration (down regulation of *ina-1*) and contributing to directionality (up-regulation of *pat-2)* of the DTC during the second migration phase (Meighan, C.M. and Schwarzbauer, J.E., 2007). This is coincidental with *unc-5* up-regulation which mediates repulsion from the ventral *unc-6* expression (Su, M. *et al.*, 2000).

Also, the differential response of Q neuroblasts to EGL-20/WNT is thought to be due to the initiation of different signaling pathways (canonical versus non-canonical) (Whangbo J., 1999). This could be the case for the DTC as it changes its response to the spatial cues between the first and the third migration phase. Since Q neuroblasts respond in a dose dependent manner perhaps the DTC is doing the same and with lower or higher concentrations of EGL-20/WNT will use alternate WNT pathways. This might also be applicable to EGL-17/FGF pathway.

6.2 EGL-20/WNT and EGL-17/FGF signaling pathways

Observations of mutant components for both EGL-20/WNT and EGL-17/FGF signaling pathways support the idea that these two signaling pathways are involved in guiding the DTC migration along the A-P axis. The different components of both EGL-20/WNT and EGL-17/FGF signaling pathways gave us the opportunity to better understand the different roles that both these cues have in guiding the DTC during the first and third migration phases. We grouped the different components in their respective signaling pathway to simplify the interactions that are taking place.

6.2.1 Egl-17/FGF components

Mutants of *clr-1* have increased signaling through EGL-15/FGFR, the only receptor for EGL-17/FGF, since CLR-1 normally represses EGL-15/FGFR. We suspect that the hyperactivity of EGL-15/FGFR for EGL-17/FGF ligand could impact the response of the DTC to the specific EGL-17/FGF gradient that is usually established at the mid-body of *C. elegans* hermaphrodites. Single mutants of *clr-1* did show some first phase late migration defects. This could be attributed to the increased sensitivity of EGL-15/FGFR for EGL-17/FGF. If EGL-17/FGF has a repulsive effect during the first migration phase, increased sensitivity of EGL-15/FGFR for EGL-17/FGF could be making the DTC migrate at a faster rate during the first migration phase. The DTC would travel farther along the A-P axis, past the reference point VD10 and DD5, before *unc-5* is up-regulated to initiate the second migration phase. We must also keep in mind that EGL-20/WNT is still present in this mutant and is attracting the DTC towards the tail of the animal. In this situation, EGL-17/FGF is conveying a stronger push than the DTC is normally used to, resulting in first phase late migrations defects. However, a hyperactive receptor could

alternatively mean that EGL-15/FGFR may not cause increase sensitivity for EGL-17/FGF but in fact could render the receptor insensitive to any gradient of EGL-17/FGF.

Double mutants of *clr-1; egl-20* displayed first phase precocious migration defects along with third phase longitudinal migration defects. Although EGL-15/FGFR might be more sensitive to EGL-17/FGF signaling, the absence of EGL-20/WNT results in the DTC migrating at a slower rate and therefore not migrating far enough by the time *unc-5* is up-regulated for the initiation of the second migration phase. This would mean that EGL-17/FGF is unable to compensate for the lack of EGL-20/WNT signaling despite the fact that they have overlapping roles in the first migration phase. We can suggest that both proteins are involved in the first migration phase since we see different migration defects in both single and double mutants of *clr-1* and *clr-1; egl-20* respectively. This could also suggest that EGL-20/WNT has a regulatory effect on the DTC's response to EGL-17/FGF. Maybe EGL-20/WNT or another element regulated by EGL-20/WNT is required to be present for the DTC to respond to EGL-17/FGF signaling. The longitudinal migrations defects in this case are only seen in the double mutant of *clr-1; egl-20* and suggest that EGL-20/WNT is responsible for this phenotype. These observations suggest that EGL-17/FGF has a smaller role in the third migration phase.

Mutations of *lin-25* also support the same model; where EGL-20/WNT has an attractive and repulsive roles in the first and third migration phase respectively and EGL-17/FGF has an repulsive and attractive role in the first migration and third migration phase respectively (Table 10). Single mutants of *lin-25* displayed first phase precocious migration defects. It was surprising to observe this phenotype in the *lin-25* mutant (an

EGL-17/FGF component) since single mutants of *egl-17* did not display any first or third phase migration defects. However, this phenotype underlines the importance of the EGL-17/FGF signaling pathway in the first migration phase of the posterior DTC (just like single mutants of *egl-20* underline EGL-20/WNT signaling pathway importance in the third migration phase).

LIN-25 is a novel nuclear protein that might act as transcription factor or part of the transcription machinery (Boyer, T.G. *et al.,* 1999; Tuck, S. and Greenwald, I., 1995). When it is mutated, it could affect gene expression that is important in regulating the first migration phase. Also *egl-17* single mutants could represent an incomplete knockout phenotype whereas *lin-25* single mutants might represent a more complete knockout and therefore have a more representative phenotype. We must also keep in mind that there are two possible ligands for EGL-15/FGFR; EGL-17/FGF and LET-756/FGF. It has been previously shown that there is some redundancy between the two ligands although their functions are very different (Fleming, T.C *et al.,* 2005). Therefore, although *egl-17* is mutated there is still some reduced signaling through the EGL-15/FGFR pathway but when *lin-25* is mutated we manage to affect the whole signaling pathway regardless of which ligand is associated with the receptor. LIN-25 could also be linked to the WNT signaling pathway and somehow when it is deleted impact both the FGF and WNT signaling pathway.

Double mutants of *lin-25; egl-20* also displayed first phase precocious migration defects along with third phase longitudinal migration defects. The slight difference in first phase precocious migration defects between the single and double mutant of *lin-25* was not significant, underlining EGL-17/FGF's major involvement in the first migration

phase. However, the difference in third phase longitudinal migrations between *lin-25* single mutants and *lin-25; egl-20* double mutants was found to be significant. This further supports that EGL-20/WNT is the main player in the third migration phase since no longitudinal migration defects were noticed in the single mutant of *lin-25*.

Finally, *lin-15* mutants also support our theory but suggest that other players are still to be identified. LIN-15 represses the development of extra vulvas along the ventral side of *C. elegans* hermaphrodites (Huang, L.S. *et al.,* 1994). The VPCs express EGL-17/FGF; therefore in *lin-15* single mutants we observe multiple vulvas as well as multiple sources of EGL-17/FGF along the ventral side of *C. elegans.* This increased EGL-17/FGF signaling disrupts the specific A-P gradient established which is reflected in the phenotypes observed for these mutant worms.

Single mutants of *lin-15* have first phase late migration defects which can be attributed to the extra EGL-17/FGF signaling, the increased amount of repulsion effect of EGL-17/FGF in the first phase pushes the DTC farther along the ventral side just like *clr-1* mutants did. The percentage of first phase late migration defects amounts to 37% (Table 6). This roughly corresponds to the number or 'extra' posterior vulvas that express *egl-17GFP* which is 21% (Section 5.7.1.6). This again indicates that EGL-17/FGF has a more significant role in the first migration phase than in the third migration phase since no third phase migration defects are noticed (Table 6).

Double mutants of *lin-15; egl-20* displayed first phase late migration defects along with third phase longitudinal migration defects. The first phase late migration defects are still likely a result of the excess of EGL-17/FGF signaling whereas the third phase longitudinal migration defects seem to be attributable to the *egl-20* mutation. Since we

believe that EGL-20/WNT is mainly responsible for repulsing the DTC back towards the dorsal mid-body, the deletion of *egl-20* usually results in longitudinal migration defects. The triple mutant of *lin-15; egl-20; egl-17* still displayed first phase late migrations and third phase longitudinal migration defects. Interestingly, had EGL-17/FGF been responsible for the first phase late migrations, we would have expected the triple mutant to display first phase precocious migration defects (lack of push from EGL-17/FGF). Instead the phenotype observed suggests that EGL-17/FGF is not completely responsible for the first phase late migration defect. The triple mutation could be affecting another factor that is essential in guiding the DTC along its first migration phase. Indeed, this would make sense since LIN-15 acts from the hypodermis and it could also be affecting the secretion of other important factors that mediate the signaling cue to the DTC (Hedgecock, E.M., 1990). Also the extra vulvas created in the *lin-15* mutants could be disrupting or creating abnormal environments which result in the phenotype observed.

6.2.2 EGL-20/WNT components

Most of the findings from EGL-20/WNT components seem to fit with the model discussed above regarding the EGL-17/FGF components. Starting with the receptors of EGL-20/WNT, MIG-1 is one of four receptors (MOM-5, LIN-17 and CFZ-2) in the WNT signaling pathway (Eisenmann, D., 2005). Single mutants of *mig-1* displayed very few first phase precocious and late migration defects and no third phase longitudinal migration defects. This is surprising since most *egl-20* mutations display third phase longitudinal migration defects. However, the WNT signaling pathway has multiple receptors and this finding suggests that, when MIG-1 is compromised, the other receptors are capable of compensating for the mutated *mig-1*. MIG-1 might be the main receptor used for EGL-20/WNT signaling for DTC migration but the other receptors of the WNT signaling pathway would have to be assayed to test this statement. The single mutant *mig-1* had 7% (N=95) of first phase precocious migration defects which could be explained by the lack of signaling through MIG-1 receptor if it was directly responsible for the first migration phase (Table 8. EGL-20/WNT components). The specific signaling of EGL-20/WNT through MIG-1 may be an important key element in regulating the first migration phase whereas one or all of the other three receptors (LIN-17, MOM-5 and CFZ-2) may be involved in the third migration phase. However, there is also an equivalent amount of first phase late migration defects (8%) in these *mig-1* single mutants. This could indicate that some of the time there is insufficient signaling due to *mig-1* mutation and in other circumstances the remaining receptors might overcompensate for the mutation during the first migration phase.

The double mutant of *mig-1; egl-17* showed an increase in frequency from the 7% (in the single mutants) to 17% in first phase precocious migration defects (Table 8). These results concur with the hypothesis that both EGL-20/WNT and EGL-17/FGF have overlapping roles involved in the first migration phase of the DTC since there is a higher frequency of these migration defects. Also worth noting is the presence of third phase longitudinal migration defects in the double mutants *mig-1; egl-17* but not in the single mutants *mig-1*. This was not expected as we thought that longitudinal migration defects were mostly linked to EGL-20/WNT signaling. We would have expected to see these longitudinal migrations defects in the single mutant *mig-1* but as we suggested earlier, the third migration phase could be linked to the remaining three WNT receptors LIN-17, MOM-5 and CFZ-2. These observations could also suggest that EGL-17/FGF does have a small role in helping the DTC in the third migration phase to migrate back to the mid-body of the animal. This is feasible although some of the *lin-15* data discussed above suggested otherwise. In the *lin-15* discussion, we suggested that EGL-17/FGF had little to do with the third phase longitudinal migration defects observed because of the lack of difference between *lin-15; egl-20* double mutant and *lin-15; egl-20; egl-17* triple mutant, particularly regarding the third phase longitudinal migration defects. However we also suggested that this phenotype could be the result of an abnormal circumstance in the triple mutants or another factor part of the signal transduction being affected. This would mask the contribution of EGL-17/FGF in regulating the third migration phase especially if it had a minor role.

We continued our study of the WNT pathway by observing single mutants of *mig-5*, a homologue of Dishevelled (downstream element of WNT signaling pathway), which displayed both first phase late migration defects and third phase longitudinal migration defects. If MIG-1 and the other WNT receptors (LIN-17, MOM-5 and CFZ-2) are involved in the first and third migration phase respectively, it is logical that this "bottle neck" factor, MIG-5, could affect both the first and third migration phase. However, this assumes that the signaling is acting through MIG-5 exclusively since there are two other Disheveled proteins in *C. elegans* (DSH-1 and DSH-2).

Also, double mutants of *mig-5; egl-17* displayed first phase precocious migrations and third phase longitudinal migration defects very similar to *egl-20; egl-17*. The change from first phase late migrations in the *mig-5* single mutants to first phase precocious migrations in *mig-5; egl-17* further suggests that EGL-17/FGF has a prominent role in guiding the DTC during the first migration phase. It also confirms that EGL-20/WNT is largely responsible for the third migration phase since the third phase longitudinal migration defects do not change significantly between *mig-5* single mutants and *mig-5; egl-17* double mutants.

Finally, EGL-5 is a transcription factor downstream in the WNT signaling pathway. Single mutants of *egl-5* did not have any significant migration defects and were very similar to wild-type *unc-5GFP* worms. Double mutants of *egl-5; egl-17* did have some migration defects characterized by first phase precocious migration defects but surprisingly, very few double mutants had third phase longitudinal migration defects. This, once again, demonstrates how both EGL-20/WNT and EGL-17/FGF have

overlapping roles in the first migration phase. It also suggests that the more upstream or downstream elements of WNT signaling pathway seem to be mainly involved mainly in the third migration phase. If LIN-17, MOM-5 and CFZ-2 receptors of WNT are responsible for the third migration phase they could be signaling through another downstream element other than EGL-5 and that would explains why we do not observe any third migration defects in the *mig-5;egl-17* double mutants. The mechanism behind the expression of *egl-5* could resemble that of *mab-5* in Q neuroblasts. In the Q neuroblasts, only the QL neuroblast which migrates into the tail of *C. elegans* expresses *mab-5* due to its proximity to EGL-20 protein. The QR, which migrates anteriorly, does not express *mab-5* at all since it is not exposed to EGL-20 signaling (Whangbo, J. and Kenyon, C., 1999).

7. CONCLUSION

In conclusion we suggest that both EGL-20/WNT and EGL-17/FGF act as guidance cues for the two antero-posterior DTC migration phases. This is analogous to the roles that EGL-20/WNT and EGL-17/FGF play in guiding the migrations of the Q neuroblasts and SMs, respectively (Silhankova, M. and Korswagen, H., 2007; DeVore D.L. *et al.*, 1995).

Previous work had indicated the involvement of SDN-1 in the proper migration of the DTC through the regulation of EGL-20/WNT. This data had also indicated the involvement of EGL-17/FGF in providing information the migrating DTCs (Schwabiuk, M., 2006). Our experiments were able to support the hypothesis that SDN-1 HSPG is required to establish a proper EGL-20/WNT gradient in the tail of *C. elegans* as well as having a role in regulating the level of expression of this protein. Both *sdn-1(ev697)* and *sdn-1(zh20)* mutant worms displayed misdistribution of EGL-20/WNT as well as increased expression.

To further investigate the mechanisms through which EGL-20/WNT and EGL-17/FGF might be working in guiding the DTC, we created single and double mutant worms of these genes. Double mutants of *egl-20; egl-17* displayed an increased frequency of migration defects in the posterior DTC characterized by a slower migration rate. We were able to show that the posterior DTC migrates at a slower rate during the first migration phase when both these guidance cues are absent. This was supported by comparing the temporal expression of *unc-5Lac-Z* in the anterior and posterior DTC. These observations indicated that despite the more anterior position of the posterior DTC along the A-P axis, the developmental timing of the expression of *unc-5* was not altered,

in the double mutants of *egl-20; egl-17*. Furthermore, we compared the gonad development relative to vulval development over time of both wild-type and double mutant of *egl-20; egl-17*. These data confirmed that the posterior DTC was moving at a slower rate during the first migration phase. Together, these data indicated that EGL-20/WNT and EGL-17/FGF do not regulate the expression of *unc-5* at the transcriptional level but that instead they seem to act as spatial guidance cues to lead the DTC along its first and third migration phase. We were able to show that both EGL-20/WNT and EGL-17/FGF have particular regions of expression which are important for the DTC to properly migrate along the first and third migration phases.

The detailed analysis of both the WNT and FGF signaling pathways has helped identify some of the individual and overlapping roles of EGL-20/WNT and EGL-17/FGF play in guiding the DTC along the first and third migration phases. Our findings indicate that EGL-20/WNT is more implicated in the third migration phase while EGL-17/FGF is more involved in the first migration phase. However, these roles are not exclusive and in both cases, EGL-20/WNT and EGL-17/FGF have overlapping roles that contribute to both the first and third migration phase. Furthermore, the phenotype of the different mutants of each signaling pathway helped confirm that EGL-20/WNT and EGL-17/FGF both alternate as attractant and repellent during the first and third migration phase. Our data also suggested that EGL-20/WNT might have a regulatory role on EGL-17/FGF function. Single and double mutants of *clr-1* indicated that the DTC might require the presence of EGL-20/WNT in order to respond to the EGL-17/FGF cue. The cooperation between the WNT and FGF signaling pathways is not uncommon. Previous work has shown that both these signaling pathways are essential and come together to regulate the

expression of Hox genes as well as to specify vulval cell fates (Eisenmann, D.M. *et al.*, 1998).

8. FUTURE DIRECTIONS

The work presented here has enabled us to further understand how EGL-20/WNT and EGL-17/FGF are involved in mediating positional information during the two A-P migrations of the DTC However; further experiments would benefit our understanding of these mechanisms. Further investigation of the link between EGL-20/WNT and EGL-17/FGF with DAF-12 would elucidate whether these spatial cues help DAF-12 up-regulate *unc-5* at the precise time in migration. To complete this analysis, single mutants of *daf-12; unc-5GFP* need to be scored and their phenotype compared to the remaining mutants.

To complete the analysis of the *unc-5(ev585)* mutants we need to score a more strains. The phenotype of the double mutants of *egl-20; unc-5(ev585)* and *egl-17; unc-5(ev585)* need to be analyzed to make conclusive remarks about these mutants.

The signaling pathways of EGL-20/WNT and EGL-17/FGF are very intricate. Although we did analyze a few of the components, many more could be tested. From these experiments not only would we get a better understanding of the mechanism of these pathways but we would also be able to pinpoint all the components involved or not in guiding the DTC. Many of these genes might require to be tested through RNAi as their phenotypes are lethal when used as null alleles. Components such as the WNT receptor have many genes that encode for different receptors. So far, we have only tested MIG-1 for its involvement in DTC guidance. It would be beneficial to test all 3 putative receptors (LIN-17, MOM-5 and CFZ-2). It would be equally beneficial to test other components such as β-catenin since the *C. elegans* also has many different genes (*bar-1*, *wrm-1*, *hmp-1* and *sys-1*) that encode this protein. The different β-catenin proteins of *C.*

elegans have been shown to have differential effects on downstream elements of the WNT signaling pathway (Korswagen, H.C., 2002).

Our experiments have showed that EGL-20/WNT and EGL-17/FGF are involved both in the first and third migration phase of the DTC. It seems that the DTC changes its response to both these spatial cues during the second migration phase enabling it to migrate in different directions along the A-P axis. Reports that both EGL-20/WNT and EGL-17/FGF are capable of assuming attractive and repulsive roles for the migration of numerous cells in *C. elegans* (Silhankova, M. and Korswagen, H., 2007; DeVore D.L. *et al.*, 1995) has suggested that this might also be the case in the migration of the DTC. However, how the DTC can change its response towards these spatial cues is still not understood. Testing different mechanism that have been previously suggested to change a cell's response to the different surrounding cues would be necessary in better understanding the regulation of DTC migration.

Investigating whether EGL-20/WNT and EGL-17/FGF components are working from within the DTC or from the surrounding tissue is also essential in completing this analysis. This has been approached using RNAi in *rde-1* mutant worms rescued with *rde-1* cDNA expressed only in the DTC. Due to time restraints we were unable to complete this work for this thesis. Elements such as LIN-15 act from the surrounding tissue and can therefore implicate many other factors in mediating information to the DTC (Herman, R.K., and Hedgecock, E.M., 1990). These experiments will be a very important piece of this story in telling us if these signaling pathways are acting cell autonomously or not.

From the work presented it is evident that EGL-20/WNT and EGL-17/FGF are not the only cues involved in DTC migration. However, further investigation of the mechanisms leading to the proper migration of the DTC along the A-P and D-V axis of *C. elegans* is sure to reveal these missing components.

9. APPENDIX

9.1 Solutions

Nematode Growth Medium Agar (NGM)	
NaCl	3g
Agar	17g
Peptone	2.5g
Cholesterol (5mg/ml in EtOH)	1ml
dH_2O	975ml
Autoclave, then add the following using sterile technique	
$CaCl_2$ 1M	1ml
$MgSO_4$ 1M	1ml
Potassium phosphate 1M pH6	25ml

LB broth	
Tryptone	10g
Yeast Extract	5g
NaCl	10g
H_2O	1L
Autoclave	

0.5M EDTA (pH 8.0)	
EDTA	14.61g
NaOH	2g
H_2O	to 80ml

50x TAE	
Tris base	242g
Glacial acetic acid	57.1ml
0.5 EDTA (pH8.0)	100ml
ddH_2O	1L
For 1x TAE, for the Agarose gel electrophoresis running buffer, add 20m 50x TAE to 1000ml dH_2O	

1% agarose gel	5mm		10mm	
	Agarose	TAE	Agarose	TAE
Small	0.2g	20ml	0.45g	45ml
Medium	0.6g	60ml	1.25g	125ml

1.5% agarose gel	5mm		10mm	
	Agarose	TAE	Agarose	TAE
Small	0.3g	20ml	0.675g	45ml
Medium	0.9g	60ml	1.875g	125ml

Ethidium Bromide (EtBr)	
EtBr	1g
dH_2O	100ml
Stir for several hours in container covered with foil, store at room temp. under fumehood.	

Orange G dye (6x loading dye)	
Glycerol	30ml
Orange G	0.25g
0.5M EDTA	400ul
dH_2O	100ml
Aliquot in 1ml eppindorf tubes and store at -20°C. While in use keep at 4°C. Add 2ul dye/10ul sample loaded.	

Low TE (10mM Tris, 1mM EDTA pH8.0)	
1M Tris pH8	1ml
0.5M EDTA, pH8	0.2ml
dH_2O	98.8ml

DNA ladder (Invitrogen)	
Orange G dye	170ul
DNA ladder	50ul
Low TE	780ul
Use no more than 10ul to load alongside samples	

LB agar	
Truptone	10g
Yeast extract	5g
NaCl	10g
Agar	15g
H_2O	1L
Autoclave and poor while still warm. If making **Amp plates**, add 2ml of 100ug/ml stock to obtain 50mg/ml concentration. For **Amp+Tet plates**, add the Amp as mentioned above and then add 1ml of 12.5mg/ml to obtain 12.5ul/ml concentration.	

RNAi plates	
Make 500ml of NGM agar, send to autoclave and add both Carbenicillin and IPTG when if has sufficiently cooled down.	
Carbenicillin (40mg/ml)	312ul
IPTG (200mg/ml)	3.75ml
OR IPTG (1M)	0.5ml

Rich agarose plates (500ml= 20 plates)	
NaCl 50mM	1.56g
Cholesterol (autoclaved) 5ul	500ul
Agarose (1.5%)	7.5g
Mix in 500ml water and autoclave. Add remaining autoclaved solutions using sterile technique.	
$CaCl_2$ 1mM	500ul
$MgSO_4$ 1mM	500ul
K-PO4 pH6.0 25mM	12.5ml

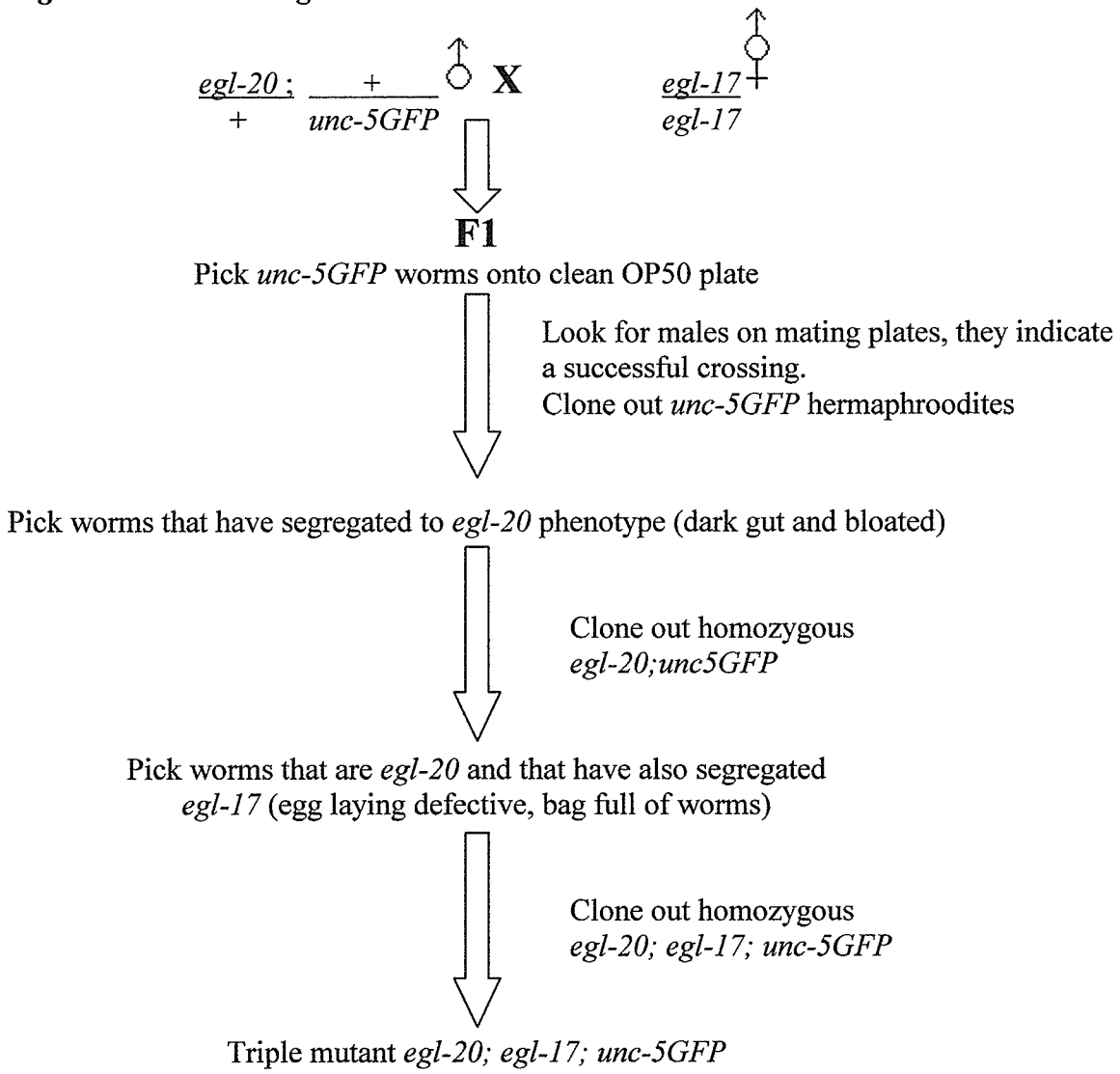
M9 buffer	
KH_2PO_4	3g
Na_2HPO_4	6g
NaCl	5g
$MgSO_4$ 1M	1ml
H_2O	1L

Genomic worm lysis solution (store at -20°C)	
Tris pH8.5 1M	1ml
NaCl 100mM	0.058g
EDTA 0.5M	1ml
SDS 10%	1ml
Beta-merccaptoethanol 1%	100ul
Proteinase K 100ug/ml	65.36ul
ddH_2O	6.2ml

10mM dNTP (Invitrogen Kit)	
dTTP 100mM	10ul
dGTP 100mM	10ul
dATP 100mM	10ul
dCTP 100mM	10ul
ddH_2O	60ul
Store at -20°C	

Single worm lysis buffer (1ml aliquots in -20oC)	
KCl 1M	0.5ml
Tris 1M	0.1ml
$MgCl_2$ 1M	0.025ml
Triton 10%	0.45ml
Tween-20 10%	0.45ml
Gelatin 10%	0.1ml
Proteinase K	30ul
ddH_2O	8.345ml

9.2 *C. elegans* mutant strain generation outlines



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