The evolution of organ shape: distal tip cell migration and gonadogenesis in nematodes.

by

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Animals display a variety of shapes in nature. The different shape of homologous organs allows them to adopt different functions and therefore, allows animals to live in different ecological niches. However, little is understood about the cellular and molecular mechanisms involved in the evolution and development of organ morphology. We compare gonad development in two closely related nematode species, *Pristionchus pacificus* and *Caenorhabditis elegans* to address how a tube can change shape. The hermaphrodite gonad in each of these species consists of two tube-shaped rotationally symmetrical gonadal arms. There is a novel ventral migration observed in *P. pacificus* that is not observed in *C. elegans*. Extension of the gonadal arms in both of these species is lead by the distal tip cell (DTC), which is a single cell that caps the end of the extending arm. Previous studies have shown that the DTCs in *P. pacificus* receive a signal from the vulva to extend ventrally during development. However, it is not clear how the DTCs are responding to dorsal/ventral positional information to accomplish this novel migration. Here, we show early data that the Netrin cell guidance cue may be involved in dorsal/ventral migrations of the *P. pacificus* DTC. Specifically, we show *Ppa-unc-40*/Netrin receptor may be a major player in dorsal/ventral migrations of the DTC.

The evolution of organ shape: distal tip cell migration and gonadogenesis in nematodes

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LIST OF ABBREVIATIONS

NGM	Nematode Growth Media	1
DTC	Distal Tip Cell	2
VPC	Vulva Precursor Cell	4
Ppa	*Pristionchus pacificus*	5
est	Expressed Sequence Tag	7
DEPC	Diethylpyrocarbonate	11
SSC	Salt Sodium Citrate	11
SSPE	Saline Sodium Phosphate Ethylene Glycol Tetra Acetic Acid	12
SDS	Sodium Dodecyl Sulfate	12
BSA	Bovine Serum Albumin	12
PBS	Phosphate Buffered Saline	12
PBTw	Phosphate Buffered Saline Tween-20	12
DAPI	4’,6-diamidino-2-phenylindole	12
EGTA	Ethylene Glycol Tetra Acetic Acid	12
HB	Hybridization Buffer	13

Introduction

Homologous vertebrate forelimbs take on different shapes that allow for different functions whether it is running, swimming, or flying through the skies (Freeman 2005). However, developmental biologists still do not fully understand how nature creates so many different shapes. For example, how has a simple tube changed shape and specialized along its axis to assume the complexities of the vertebrate heart (Bishopric 2005; Olson 2006)? With the complexity of these sophisticated organs, we need simple comparative models of organ shape change that are distant enough to have observable difference, yet closely related enough to have homologous mechanisms. We have chosen the hermaphrodite gonad of two closely related nematode species, *Pristionchus pacificus* and *Caenorhabditis elegans* to address how a tube can change shape.

P. pacificus diverged from *C. elegans* about 300 million years ago (Pires-daSilva and Sommer 2004). The *Pristionchus* genus is part of the Diplogastridae family, while *C. elegans* is part of the Rhabditidae family (Meyer et al. 2007 and 2009). Nematode species have proven to be exceptional model organisms in the lab. They are easily maintained on Nematode Growth Media (NGM) plates, which are easy to store and do not take up much space. They are fed using a laboratory strain of *Escherichia coli* (OP50). The *C. elegans* genome is fully sequenced (Hillier et al. 2005) and a large portion of the *P. pacificus* genome is sequenced. We know the cell lineage for these species and we can utilize forward and reverse genetic approaches. Both species are transparent, making observation of the internal organs, such as the gonad, simple using a microscope. The *C. elegans* gonad is well described and has served as a model for cellular studies in numerous experiments including cell fate specification, morphogenesis, cell signaling, cell cycle control, and programmed cell death (Hubbard and Greenstein 2000). *P. pacificus* has

many differences when compared to *C. elegans* including the timing of developmental events (fig. 1B), composition and morphology of tissues (fig. 1A), cell signaling between the somatic gonad and germ line, and has been used in a number of comparative studies with *C. elegans* including studies of vulva development and gonad development (Jungblut et al. 2001; Rudel et al. 2005; Rudel et al. 2008). These characteristics make the nematode gonad an excellent model to study the cellular and molecular processes involved in determining how a tube can change shape.

The Hermaphrodite Gonad

The hermaphrodite gonad of *C. elegans* consists of two tube-shaped rotationally symmetrical gonadal arms. Each arm is a complete ovo-testie with the somatic tissue arranged on the outside of the arm and the germ line on the inside (fig. 1A). The extension of both arms starts at the anterior-posterior center of the animal along the ventral body wall and this extension is lead by the distal tip cells (DTCs). The DTC is a single cell that caps the distal end of each arm and serves as a germ line stem cell niche for the extending gonadal arm. One arm extends towards the anterior and one arm extends towards the posterior. After extending along the ventral body wall, both arms turn and begin extending towards the dorsal body wall. After reaching the dorsal body wall, both arms turn and begin extending back towards the anterior-posterior center along the dorsal body wall. The arms eventually reach the anterior-posterior center thus giving the arms a “u” shape (fig. 1C). Extension of the arms occurs in pre-defined stages of the life cycle and the extension along the dorsal body wall back towards center occurs during the L4 larval stage, the last larval stage before the nematode developments into an adult (fig. 1B).

Like *C. elegans,* the hermaphrodite gonad of *P. pacificus* consists of two tube-shaped gonadal arms and begins its development similarly to *C. elegans*. The arms begin at the anterior-

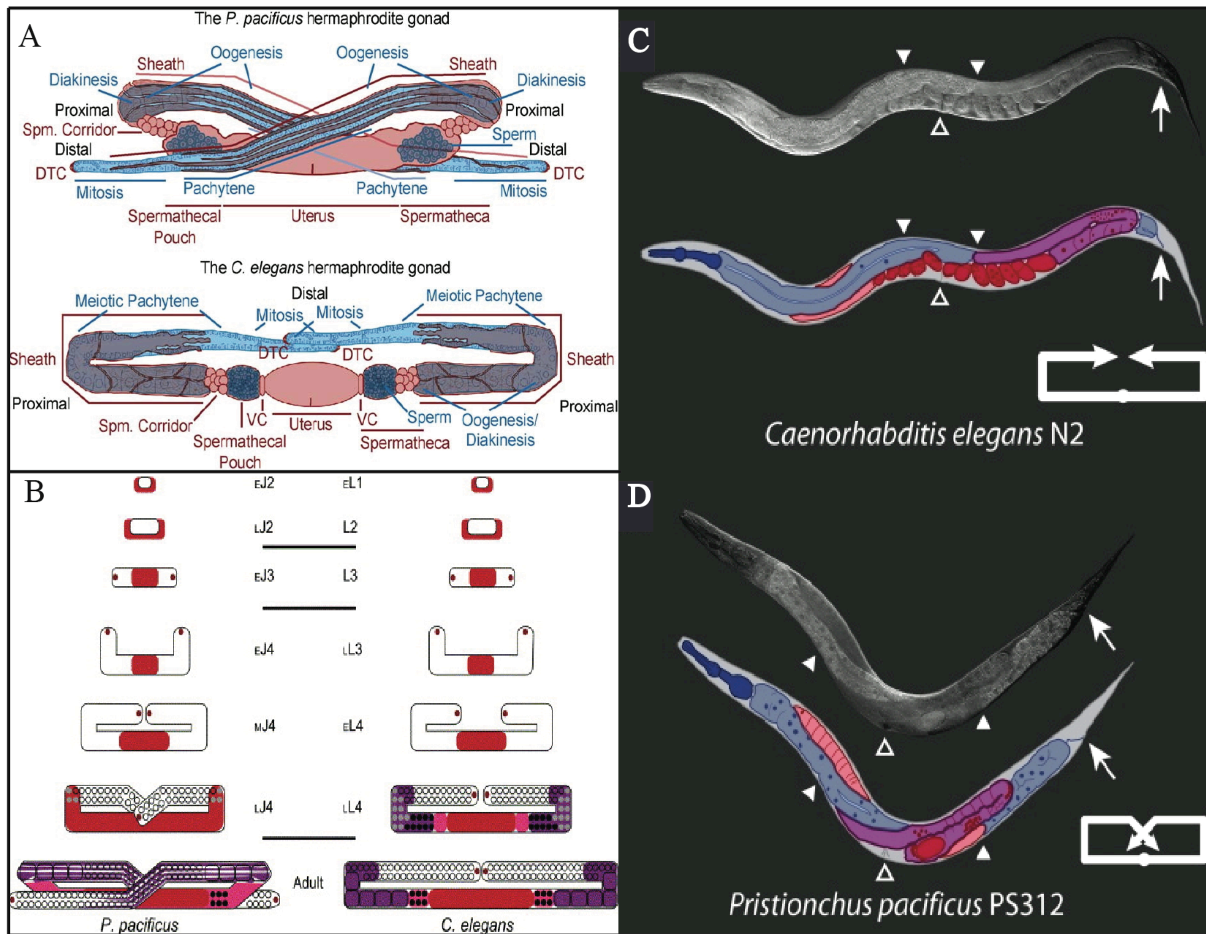


Figure 1: The adult hermaphrodite gonad of *P. pacificus* and *C. elegans*. (A) Somatic tissues are shown in red and germ line tissues are shown in blue. Note the difference in shape as well as the arrangement of cell, although the experiments in this paper will only discuss the difference in shape. (B) Developmental timing of the hermaphrodite gonad of *P. pacificus* and *C. elegans. P. pacificus* gonad development is shown on the left through larval stages J2-J4 (J1 occurs pre-hatching and only post-hatching stages are shown). *C. elegans* gonad develop is shown on the right through larval stages L1-L4. E denotes early, M denotes middle, and L denotes late. Uterus is shown in red and DTCs are shown as dark red dots. Sheath is shown in purple and spermatheca in pink. Open circles show germ cell nuclei and closed circles show spermatocyte nuclei. (C-D) Nomarski photographs and color-coded cartoons of adult *C. elegans* and *P. pacificus* hermaphrodites. Anterior is to the left, posterior is to the right, ventral is towards the bottom, and dorsal is towards the top. Somatic gonad and germ line are in purple and red. The gut is in blue. Solid white triangle shows position of the DTCs. Open white triangle shows position of the vulva. White arrow shows position of anus for purposes of identifying the ventral side. (adapted from Rudel et al. 2005 and 2008)

posterior center and extend along the ventral body wall, although they do not extend as far as they do in *C. elegans* along the ventral body wall, before they turn towards the dorsal body wall. After reaching the dorsal body wall the arms extend back towards the anterior-posterior center. However, during their return, they extend back to the ventral body wall giving the arms a pretzel

shape (fig. 1A). This return to the ventral body wall occurs during the J4 larval stage (fig. 1D. (Rudel et al. 2005). The goal of this study is to use these two nematode gonads as a model to address how a tube can change shape.

Previous laser ablation experiments have shown that the DTCs require a signal from the vulva to cue their ventral extension (Rudel et al. 2008). When P(5-7).p, the vulval precursor cells (VPCs) are ablated, the arms never extend back to the ventral body wall in the absence of the vulva. Instead they are "u" shaped like *C. elegans*. The signal is quantitative as seen when the VPCs are ablated as pairs, the arms only extend ventrally 46% of the time compared to 69% in wild type animals. There is no affect when only a single VPC is ablated or when many other tissues are ablated (Rudel et al. 2008). Ventral migration can also be disrupted with genetic ablation of the vulva (Rudel et al. 2008).

<u>*Canonical Wnt Signal*</u>

A canonical Wnt signal mediates communication between the vulva and the DTCs. The canonical Wnt pathway is one of a small number of signaling pathways conserved throughout animal development (Eisenmann 2005; Pires-daSilva and Sommer 2003). In the absence of a Wnt signal, β-catenin is found in the cytoplasm and is bound by a multi-protein destruction complex formed by APC, Axin, CKI, and GSK3β. This destruction complex phosphorylates β-catenin, which marks it for degradation by the proteosome. In this situation β-catenin does not move into the nucleus (fig. 2). In the presence of a Wnt signal, a Wnt ligand binds to a Frizzled family receptor and a coreceptor, LRP-5/6/arrow family. There are multiple Wnt ligands (*lin-44*, *egl-20*, *mom-2*, *cwn-1*, and *cwn-2*) and multiple Wnt receptors (*lin-17*, *mom-5*, *mig-1*, and *cfz-2*). Wnt ligand binding inhibits the destruction complex from phosphorylating β-catenin, therefore, allowing it to move into the nucleus where it binds to TCF/LEF-1, a DNA binding protein. When

β-catenin interacts with TCF/LEF-1 it initiates a change in target gene transcription (fig. 2). TCF/LEF, which acts as a repressor on its own, is transformed upon β-catenin binding into an activator (Eisenmann 2005).

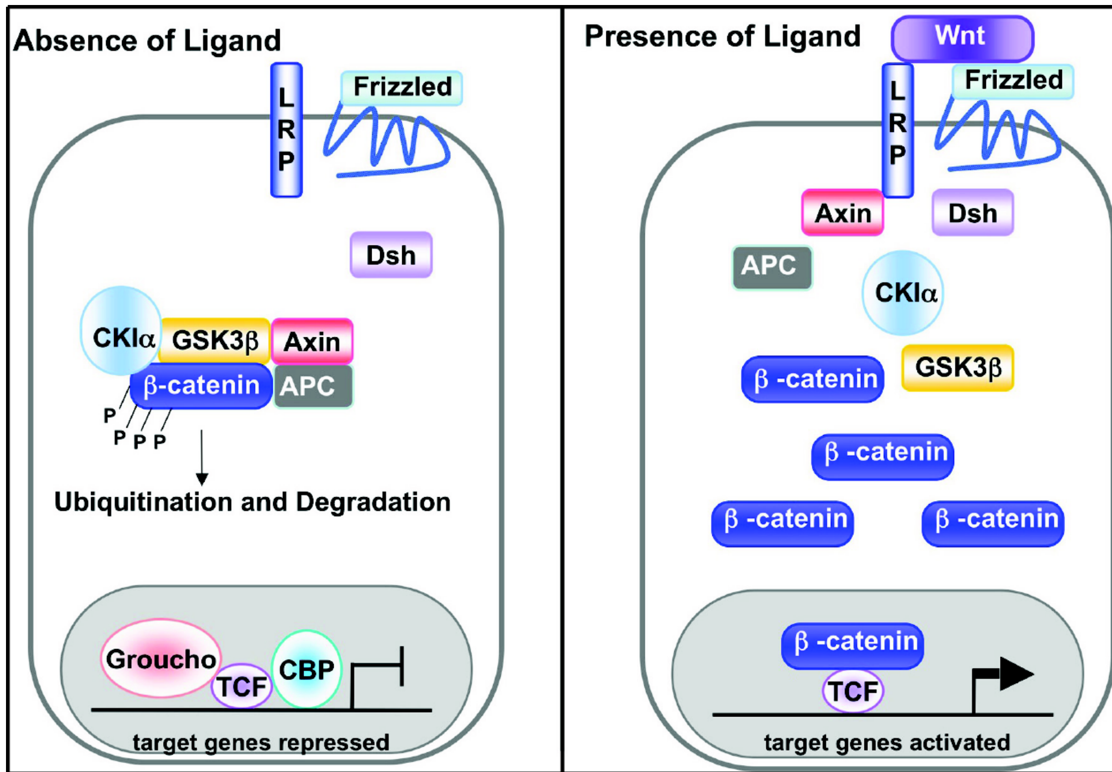


Figure 2: Canonical Wnt pathway (from Eisenmann 2005)

The Wnt pathway triggers the novel ventral migration in *P. pacificus*. *Ppa*-BAR-1 is a homologue of β-catenin and is expressed in the nuclei of the DTCs during the J4 larval stage when ventral gonadal arm extension is observed. *Ppa*-BAR-1 has also been shown to be necessary for ventral migration; in *Ppa-bar-1* mutants gonadal arms do not extend ventrally. However, *Ppa-bar-1* mutants fail to form a vulva, therefore it is not clear whether the arm's failure to extend ventrally is due to the absence of *Ppa-bar-1* or the absence of a vulva. In Wnt ligand mutants *Ppa-mom-2* and *Ppa-lin-44*, animals do form a normal vulva and show a reduction in gonadal arm ventral extension, although the reduction is not as strong as seen in

Ppa-bar-1 mutants. Furthermore, based on RNA *in situ* hybridization experiments in whole-mount animals *Ppa-mom-2* and *Ppa-lin-44* are expressed in the vulva during the J4 developmental stage when ventral gonadal arm extension begins (Rudel et al. 2008).

<u>*Netrin Cell Guidance Cue*</u>

Our goal is to determine how the Wnt signal from the vulva instructs distal tip cell migration. In other words, how do the DTCs interpret dorsal/ventral positional information once the Wnt signaling pathway has been triggered? Our working hypothesis is that the Wnt signal allows the distal tip cells to recognize the Netrin cell guidance cue. Netrin is a conserved pathway in metazoans (Wadsworth 2002) and is involved in all post-embryonic dorsal/ventral migrations in *C. elegans* (Culotti and Merz 1998; Livesey and Hunt 1997; Lehmann 2001; Hedgecock et al. 1990). During development of the gonad in *C. elegans* Netrin mutants, the gonadal arms still extend out towards the anterior or posterior ends, respectively, turn, and return to center forming a "u" shape; however, they do so completely on the ventral side of the animal (fig. 3) (Su et al. 2000).

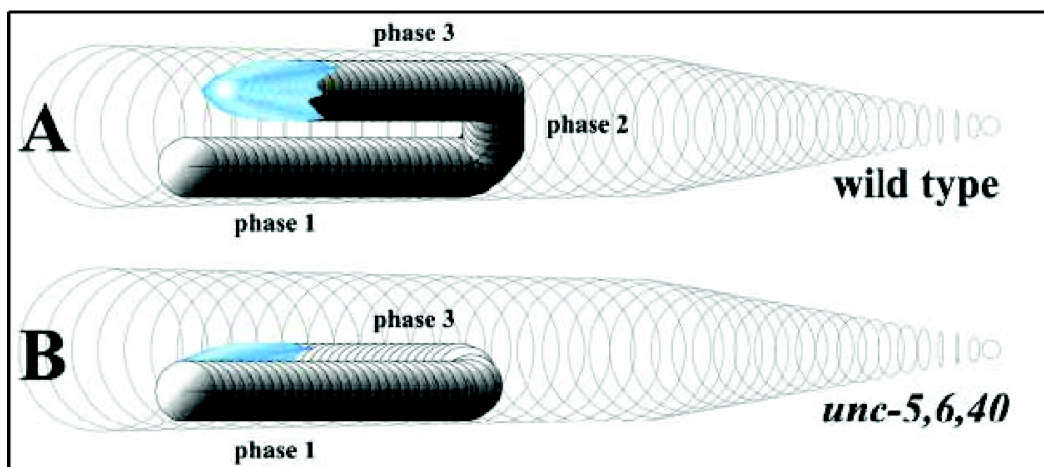


Figure 3: Cartoon of *C. elegans* hermaphrodite gonad showing wild type and Netrin mutant gonad development. Dorsal is towards the top and ventral is towards the bottom bottom. Anterior is to the left, posterior to the right. Only one arm is shown. DTCs are colored in blue. (from Su et al. 2000)

There are three principle players involved in the Netrin cell guidance cue. UNC-6 is the diffusible Netrin ligand and is believed to exist in a gradient with a higher concentration on the ventral side and a lower concentration on the dorsal side. The structurally unrelated UNC-5 and UNC-40 Netrin receptors bind Netrin and allow recognition of the graded cue (fig. 4). When UNC-5/Netrin receptor is expressed alone, it mediates a repulsive cue away from UNC-6/Netrin and therefore dorsal migration. This also occurs if UNC-5/Netrin receptor is expressed with UNC-40/Netrin receptor. If UNC-40/Netrin receptor is expressed alone, it mediates an attractive cue towards UNC-6/Netrin and therefore ventral migration (Hedgecock et al. 1990). Manipulation of Netrin receptor expression by rescuing *unc-5* mutants with an *unc-5* transgene has been shown to be sufficient to initiate dorsal migrations in DTCs (Su et al. 2000). Interestingly, in *C. elegans*, mutations in Wnt genes affect some of the Netrin dependent dorsal/ventral migration in neuronal cells/axons (Silhankova and Korswagen 2007; Killeen and Sybingco 2008).

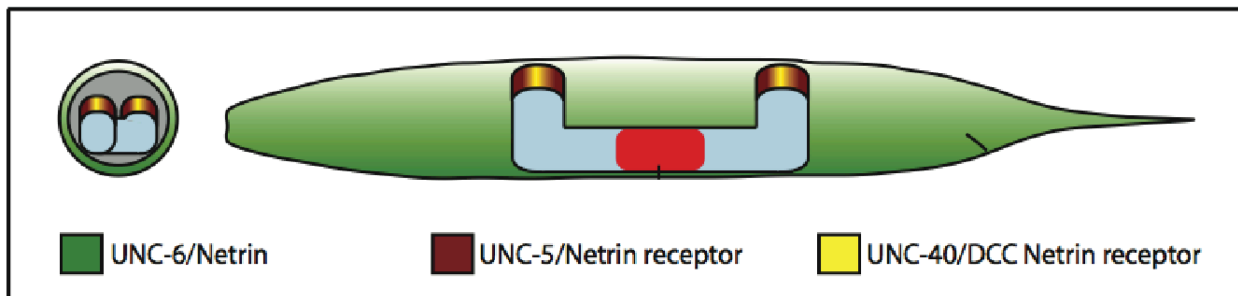


Figure 4: Cartoon of Netrin and Netrin receptors as they are orientated in nematodes. Anterior is to the left, posterior is to the right, ventral is towards the bottom, and dorsal is towards the top. Green gradient shows UNC-6/Netrin throughout the nematode's body. UNC-5 and UNC-40 Netrin receptors are shown on the DTCs. Red shows the uterus. (Rudel unpublished)

C. elegans has copies of UNC-6/Netrin and both Netrin receptors, UNC-5 and UNC-40. Interestingly, even with greater than 250-fold coverage of the *Pristionchus* genus genome and many est libraries, no UNC-5 homologue has been found. It is possible the UNC-5/receptor is

absent or has changed to such a degree that we can no longer easily identify it. UNC-5 and UNC-40 act together to signal dorsal extension in *C. elegans*. Furthermore, we have not found a homologue for UNC-129/TGF-β. UNC-129/TGF- β acts with UNC-5 to enhance UNC-5+UNC-40 signaling (MacNeil et al. 2009). The absence of both UNC-5 and UNC-129 may indicate the loss of UNC-5 from the *Pristionchus* genome. Thus, in *P. pacificus* there may only be a single Netrin receptor, UNC-40, to interpret the UNC-6/Netrin gradient. Either way, it is likely the Netrin cell guidance cue is interpreted differently in the *Pristionchus* genus. One possibility for how UNC-40/Netrin receptor interpreting the Netrin cell guidance cue differently is that UNC-40 may respond to the two UNC-6/Netrin like guidance cues present in the *P. pacificus* genome to compensate for the lack of UNC-5/Netrin receptor. The other possibility is that the presence or absence of UNC-40/Netrin receptor expression is responsible for proper dorsal/ventral cell

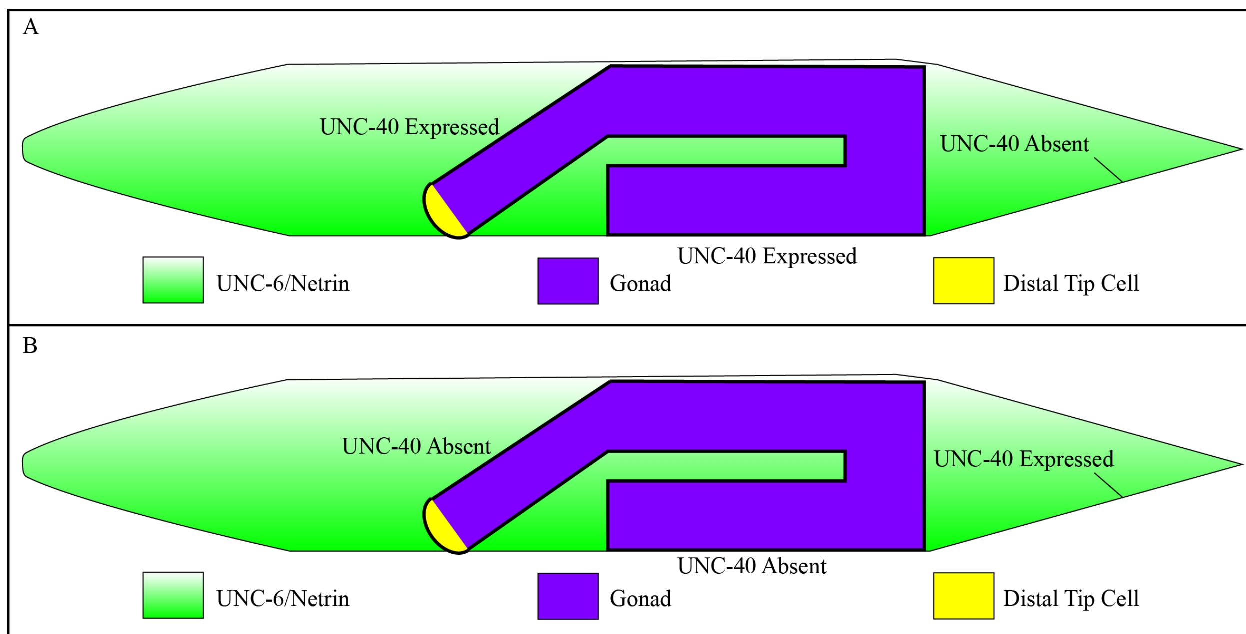


Figure 5: Cartoon representing the possible models for *unc-40*/Netrin receptor expression or absence on the DTC to allow proper recognition and response to the UNC-6/Netrin gradient during gonadogenesis in *P. pacificus*. Anterior is to the left, posterior is to the right, ventral is towards the bottom, and dorsal is towards the top. Note only one arm is shown. (A) Model representing a scenario in which *unc-40* is involved in ventral attraction as it is in *C. elegans*. (B) Model representing a scenario in which *unc-40* is involved in dorsal extension.

migration responses to the UNC-6/Netrin guidance cue in *Pristionchus*. We propose that the DTCs respond to the Wnt signal from the vulva by altering the expression of the UNC-40 Netrin receptor on the DTC surface to respond to the UNC-6/Netrin gradient (fig. 5).

Materials and Methods

Strain Maintenance

All strains were maintained at 20°C as described by S. Brenner (1974). *P. pacificus* strain PS312 was used for sequence analysis, Northern blotting, and RNA *in situ* hybridization. *P. pacificus* strains PS1843 and JU723 were also used in Northern blot analysis.

Sequence Analysis

Sequence comparisons for *P. pacificus* and *C. elegans* were obtained from pristionchus.org and wormbase.org respectively. Pristionchus assembly freeze 1 was used for blast comparisons. Intron/exon boundaries in the predicted sequence were preliminarily inferred based on blast analysis and the *C. elegans* splice site consensus sequences GUAAGUU and UUUUCAG were used to predict the location of 3' and 5' splice sites. The cDNA sequences for *unc-40* and *pop-1* were confirmed by using reverse transcription PCR to amplify cDNA from total RNA using Promega GoTaq Flexi Polymerase kit. Sequence data was analyzed using the DNA* software package. The 5' end was confirmed with a SL1 primer, which is a trans-splice leader sequence that is not associated with the gene, but is found at the beginning of ~70% of genes in *C. elegans* and is conserved in *P. pacificus* (Blumenthal 2012; Guiliano and Blaxter 2006; Lee and Sommer 2003). To confirm the 3' end of both *unc-40* and *pop-1* circularized mRNA was used as a template. Circularized mRNA was obtained as described by Contreras et al. 2011. PCR products were sequenced using BigDye Terminator.

Digoxygenin Labeled Probe Generation

A template for generation of the probe was made with reverse transcription PCR using a Promega GoTag Flexi Polymerase kit. The template for the antisense probe was made using the forward primer TGGAATGCTATTCATCTGGAC and the reverse primer

taatacgactcactatAGGGGGTACCATACAACGAACT (the lower case letters represent the region the T7 polymerase will bind to during the probe generation reaction). The sense probe was made using the forward primer taatacgactcactatagGGAATGCTATTCATCTGGACG (the lower case letters represent the region the T7 polymerase will bind to during the probe generation reaction) and the reverse primer AGGGGGTACCATACAACGAAC. The PCR product was cleaned using a Promega Wizard SV Gel and PCR Clean-Up System kit. The cleaned PCR products were used as a template for probe generation using an Ambion Megascript T7 kit and Roche DIG Labeled RNA Mix. The probes were cleaned by a lithium chloride precipitation followed by an ethanol precipitation.

Northern Blot

12µg of total RNA from *P. pacificus* strains PS312, PS1843, and JU723 were mixed with 2 µl of 5x formaldehyde gel running buffer (0.1 M MOPS pH 7.0, 40 mM sodium acetate, and 5 mM EDTA pH 8.0), 3.5 µl of formaldehyde and, and 10 µl of formamide. The samples were incubated at 65°C for 15 minutes then cooled on ice. RNA was separated on a 1% gel. The 1% gel was prepare by melting the appropriate amount of agarose in DEPC treated water, then adding 5x formaldehyde gel-running buffer to give a final concentration of 1x and formaldehyde to give a final concentration of 2.2M. All washes were at room temperature unless otherwise indicated. After electrophoresis was stopped, the gel was washed 4 times in DEPC treated water for 15 minutes per wash. Nucleic acids were then transferred to a nylon membrane as follows: The membrane was conditioned by wetting in DEPC treated water followed by soaking in 20x SSC transfer buffer for 5 minutes. A stack was fabricated from 20 sheets of GB004 blotting paper, 4 sheets of dry 3MM Chr blotting paper, 1 sheet of 3MM Chr blotting paper pre-wet with transfer buffer, the pre-treated membrane, the gel, 3 sheets of 3MM Chr blotting paper presoaked

in transfer buffer, and a buffer wick presoaked in transfer buffer. Transfer was conducted for 9 hours. The membrane was washed in 2x SSC for 5 minutes. The RNA was UV cross-linked to the membrane and the blot allowed to dry. Prior to hybridization the membrane was soaked in 6x SSPE for 2 minutes. The membrane was prehybridized in hybridization buffer consisting of 50% formamide, 6x SSPE, 5x Denhardt's reagent, 0.5% SDS, and 100 µg/ml yeast RNA for 1 hour at 42°C. Then the membrane was incubated in hybridization buffer with 1 µg of digoxygenin labeled probe for 24 hours at 42°C. Following hybridization the membrane was washed in 2x SSPE and 0.5% SDS for 5 minutes, 2x SSPE and 0.1% SDS, and in 0.1x SSPE and 0.5% SDS for 30 minutes at 37°C with gentle agitation. The wash solution was then replaced with fresh 0.1x SSPE and 0.5% SDS and incubated for 30 minutes at 62°C with gentle agitation and briefly washed in 0.1x SSPE. For visualization of the transcript, the membrane was incubated in 0.5% BSA in PBS for 30 minutes at room temperature in alkaline-phosphatase-conjugated anti-DIG antibody diluted 1:1000 (BSA/PBTw) overnight at 4°C, and washed four times in PBTw for 15 minutes each. The membrane was stained with a solution consisting of 1 BCIP/NBT tablet dissolved in 10 ml water with 0.1% tween-20, 1 mM levamisole and 1 µg/ml DAPI. Once a color change was observed, reactions were stopped with three 5 minute PBTw washes.

RNA in situ hybridization

Whole-mount RNA in situ protocol #1

Reactions were performed in 1.5 ml eppendorf tubes. The animals were fixed with a 10 minute incubation in methanol at -20°C, a 10 minute incubation in acetone at -20°C, and a 30 minute incubation in a fixation solution consisting of 1x PBS with 0.08 HEPES (pH 6.9), 1.6 mM $MgSO_4$ 0.8 mM ethylene glycol tetra acetic acid (EGTA) and 3.7% formaldehyde at room temperature. Cracks were created in the cuticle by performing five freeze-thaw cycles which

consisted of switching between liquid nitrogen and a 50°C water bath. Fixed animals were washed three times in PBS with 0.1% tween-20 for 5 minutes per wash at room temperature. The fixed animals were then treated with a 15 minute incubation in 20 μg/ml Proteinase K at room temperature. The Proteinase K treatment was stopped with a 5 minute wash in 2 mg/ml Glycine at room temperature. The animals were prehybridized in hybridization buffer consisting of 5x SSC, 50% deionized formamide, 100 μg/ml total yeast RNA, 50 μg/ml Heparin and 0.1% tween-20 for 1 hour at 48°C. A single stranded digoxygenin labeled RNA probe was generated from reverse transcribed cDNA and boiled in hybridization buffer for one hour and cooled to 48°C. The hybridization buffer used for prehybridization was removed and replaced with hybridization buffer with 1μg of digoxygenin labeled RNA probe. Animals were hybridized for 24 hours at 48°C. Following hybridization the animals were washed in HB:PBTw gradients; 5:0, 3:2, 1:4, 0:5 for 15 minutes per wash at 48°C. Three final washes were performed in PBTw for 15 minutes per wash at room temperature. For visualization of hybridization, the animals were incubated in 0.5% BSA in PBS for 30 minutes at room temperature, in alkaline-phosphatase-conjugated anti-DIG antibody diluted 1:1000 (BSA/PBTw) overnight at 4°C, and washed four times in PBTw for 10 minutes per wash at room temperature. The animals were stained with a solution consisting of 1 BCIP/NBT tablet dissolved in 10 ml water with 0.1% tween-20, 1 mM levamisole and 1 μg/ml DAPI. Once a color change was observed, the reactions were stopped with two 5 minute PBTw washes.

Whole-mount RNA in situ protocol #2

Animals were placed in a drop of PBS on a poly-L-lysine subbed slide. A cover slip was placed over the animals and the excess liquid was removed by wicking it from the edge of the cover slip with a kim-wipe. A 26-gauge needle was used to gently press down repeatedly (about

25-30 times) on the cover slip over each nematode. The pressure of the needle on the cover slip causes the bodies to expand and contract creating micro-fissures in the tissues thus aiding tissue permeabilization. The slide was placed on dry ice for approximately 10 minutes after which the cover slip was removed quickly before the slide was able to thaw. All solutions were placed on the slide and removed by wicking the liquid off with a kim-wipe. The animals were fixed with a 10 minute incubation in methanol at -20°C, a 10 minute incubation in acetone at -20°C, and a 30 minute incubation in a fixation solution consisting of 1x PBS with 0.08 HEPES (pH 6.9), 1.6 mM $MgSO_4$ 0.8 mM ethylene glycol tetra acetic acid (EGTA) and 3.7% formaldehyde at room temperature. Fixed animals were washed three times in PBS with 0.1% tween-20 for 5 minutes per wash at room temperature. The fixed animals were then treated with a 15 minute incubation in 20 μg/ml Proteinase K at room temperature. The Proteinase K treatment was stopped with a 5 minute wash in 2 mg/ml Glycine at room temperature. The animals were prehybridized in hybridization buffer consisting of 5x SSC, 50% deionized formamide, 100 μg/ml total yeast RNA, 50 μg/ml Heparin and 0.1% tween-20 for 1 hour at 48°C. A single stranded digoxygenin labeled RNA probe was generated from reverse transcribed cDNA and boiled in hybridization buffer for one hour and cooled to 48°C. The hybridization buffer used for prehybridization was removed and replaced with hybridization buffer with 1μg of digoxygenin labeled RNA probe. Animals were hybridized for 24 hours at 48°C. Following hybridization the animals were washed in HB:PBTw gradients; 5:0, 3:2, 1:4, 0:5 for 15 minutes per wash at 48°C. Three final washes were performed in PBTw for 15 minutes per wash at room temperature. For visualization of hybridization, the animals were incubated in 0.5% BSA in PBS for 30 minutes at room temperature, in alkaline-phosphatase-conjugated anti-DIG antibody diluted 1:1000 (BSA/PBTw) overnight at 4°C, and washed four times in PBTw for 10 minutes per wash at room temperature.

The animals were stained with a solution consisting of 1 BCIP/NBT tablet dissolved in 10 ml water with 0.1% tween-20, 1 mM levamisole and 1 μg/ml DAPI. Once a color change was observed, the reactions were stopped with two 5 minute PBTw washes.

RNA in situ protocol for dissected gonads

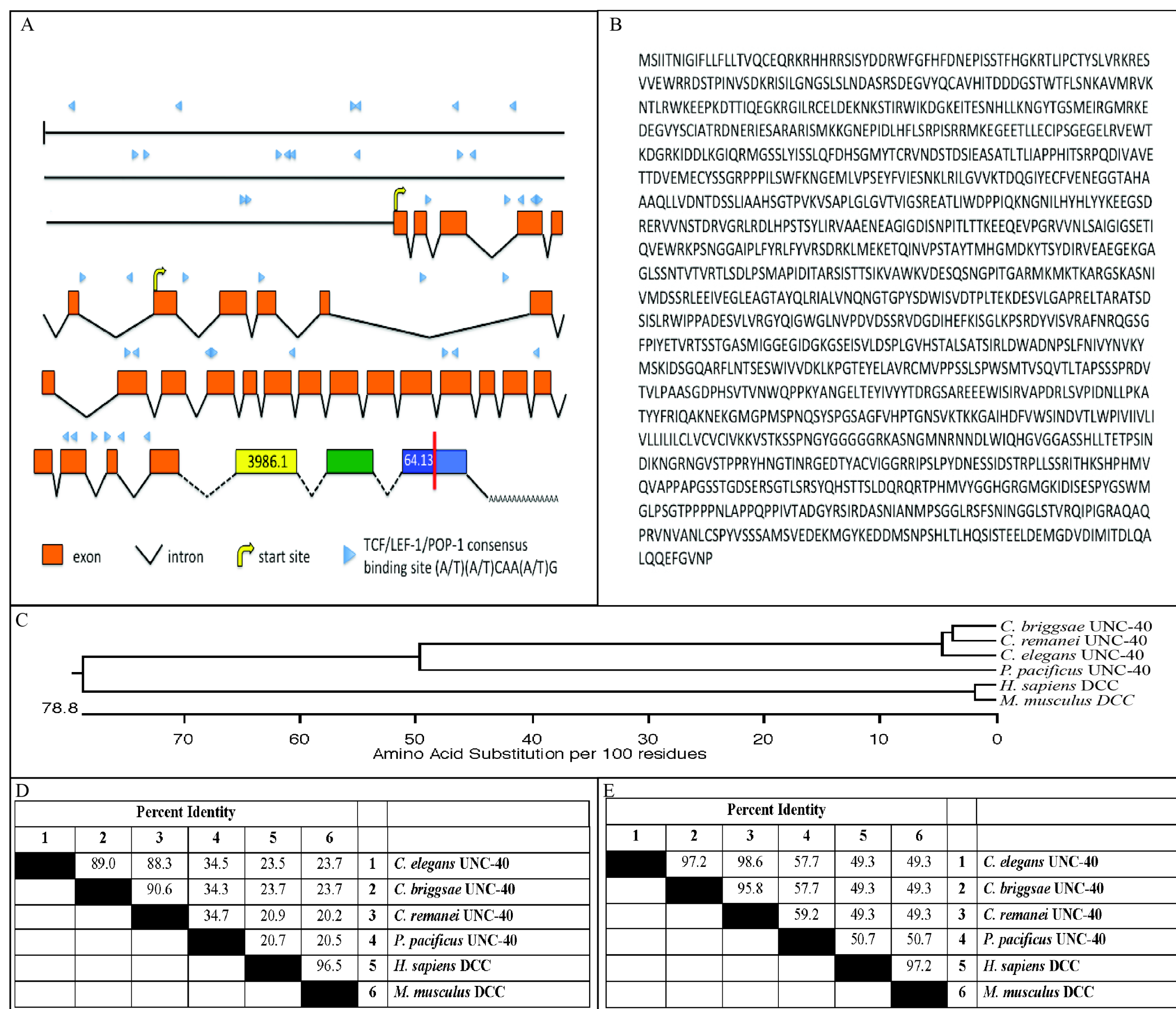
The gonads were extruded out of the animal's body by cutting off the head at the base of the pharynx with a 26-gauge needle in 0.25% levamisole in PBS. The extruded gonads and the attached bodies were transferred to a poly-L-lysine subbed slide. A cover slip was placed over the dissected gonads and the excess liquid was removed by wicking it from the edge of the cover slip with a kim-wipe. A 26-gauge needle was used to gently press down repeatedly (about 25-30 times) on the cover slip over each dissected gonad. The pressure of the needle on the cover slip causes the gonads and bodies to expand and contract creating micro-fissures in the tissues thus aiding tissue permeabilization. The slide was placed on dry ice for approximately 10 minutes after which the cover slip was removed quickly before the slide was able to thaw. All solutions were placed on the slide and removed by wicking the liquid off with a kim-wipe. The dissected gonads were fixed with a 10 minute incubation in methanol at -20°C, a 10 minute incubation in acetone at -20°C, and a 30 minute incubation in a fixation solution consisting of 1x PBS with 0.08 HEPES (pH 6.9), 1.6 mM $MgSO_4$ 0.8 mM ethylene glycol tetra acetic acid (EGTA) and 3.7% formaldehyde at room temperature. Fixed dissected gonads were washed three times in PBS with 0.1% tween-20 for 5 minutes per wash at room temperature. The fixed dissected gonads were then treated with a 7.5 minute incubation in 20 μg/ml Proteinase K at room temperature. The Proteinase K treatment was stopped with a 5 minute wash in 2 mg/ml Glycine at room temperature. The dissected gonads were prehybridized in hybridization buffer consisting of 5x SSC, 50% deionized formamide, 100 μg/ml total yeast RNA, 50 μg/ml Heparin and 0.1%

tween-20 for 1 hour at 48°C. A single stranded digoxygenin labeled RNA probe was generated from reverse transcribed cDNA and boiled in hybridization buffer for one hour and cooled to 48°C. The hybridization buffer used for prehybridization was removed and replaced with hybridization buffer with 1μg of digoxygenin labeled RNA probe. Dissected gonads were hybridized for 24 hours at 48°C. Following hybridization the dissected gonads were washed in HB:PBTw gradients; 5:0, 3:2, 1:4, 0:5 for 15 minutes per wash at 48°C. Three final washes were performed in PBTw for 15 minutes per wash at room temperature. For visualization of hybridization, the dissected gonads were incubated in 0.5% BSA in PBS for 30 minutes at room temperature, in alkaline-phosphatase-conjugated anti-DIG antibody diluted 1:1000 (BSA/PBTw) overnight at 4°C, and washed four times in PBTw for 10 minutes per wash at room temperature. The dissected gonads were stained with a solution consisting of 1 BCIP/NBT tablet dissolved in 10 ml water with 0.1% tween-20, 1 mM levamisole and 1 μg/ml DAPI. Once a color change was observed, the reactions were stopped with two 5 minute PBTw washes.

Results

Ppa-unc-40 Sequence Analysis

The cDNA sequence for *unc-40* was confirmed by reverse transcription PCR. Intriguingly, we found two SL1 splice forms suggesting two transcripts with alternative 5' ends. The cDNA sequence for the longer transcript is 4740 nucleotides long. In the genomic sequence, there are 603 base pairs between the first SL1 splice site and the second. The majority of the genomic sequence was found in Contig64.16 based on blast searches with the genome assembly found on www.pristioncus.org. However, the 3' end showed an exon found in Contig3986.1, an un-integrated sequence read, and an exon found in Contig64.13 (fig. 6A). Within the genomic sequence, the *pop-1* binding sites based on the consensus sequence (A/T)(A/T)CAA(A/T)G are found 5 times more than would be expected to occur by chance alone (fig. 6A). The overall of the immunoglobulin repeats found prominently in the UNC-40 protein, there is 57.7% identity between *P. pacificus* and *C. elegans* (fig. 6E). The high identity and placement within a protein family tree (fig. 6C) suggests the sequence we found is *Ppa-unc-40*.



D

Percent Identity							
1	2	3	4	5	6		
	89.0	88.3	34.5	23.5	23.7	1	*C. elegans* UNC-40
		90.6	34.3	23.7	23.7	2	*C. briggsae* UNC-40
			34.7	20.9	20.2	3	*C. remanei* UNC-40
				20.7	20.5	4	*P. pacificus* UNC-40
					96.5	5	*H. sapiens* DCC
						6	*M. musculus* DCC

E

Percent Identity							
1	2	3	4	5	6		
	97.2	98.6	57.7	49.3	49.3	1	*C. elegans* UNC-40
		95.8	57.7	49.3	49.3	2	*C. briggsae* UNC-40
			59.2	49.3	49.3	3	*C. remanei* UNC-40
				50.7	50.7	4	*P. pacificus* UNC-40
					97.2	5	*H. sapiens* DCC
						6	*M. musculus* DCC

Figure 6: *Ppa-unc-40* sequence. (A) Representation of the genomic sequence. The boxes represent exons and solid lines represent introns with confirmed sequences. The dashed lines represented incompletely sequenced introns. We are currently working on confirming the sequences for these introns. The vertical red line in last exon represents the position of the stop codon. The yellow, green, and blue squares represent the exons not found in Contig64.16. (B) Protein sequence. (C) UNC-40 phylogenetic tree. (D) Overall similarity of the UNC-40 protein (E) Similarity of the one of the UNC-40 immunoglobulin I-set domains.

Ppa-unc-40 Northern Blot and RNA *in situ* hybridization

A *Ppa-unc-40* digoxygenin labeled probe was generated from reverse transcribed cDNA and covered the middle region of the *unc-40* gene from exon 11 to exon 22. The probe was tested with a Northern blot using total RNA extracted from *P. pacificus* strains PS312, PS1843, and JU723. A diffuse band that is about the right size (4740 base pairs) is observed (fig. 7). Given the

possibility there are two *unc-40* transcripts, this single band may be the result of poor resolution on the gel. Perhaps a higher percentage gel would show a separation. Also, there may be a difference in the relative levels of the transcripts and perhaps one is not abundant enough to show up on the gel. Furthermore, there appears to be a difference in the intensity of the bands when comparing the bands for the three different strains, suggesting there may be a correlation between the amount of *Ppa-unc-40* transcript that is present and the percentage of the gonadal arms that extend ventrally. Ventral extension of the gonadal arms is observed approximately 70% of the time in PS312, 20% of the time in PS1843, and 97% of the time in JU723. Suggesting the UNC-40/Netrin receptor and thus UNC-6/Netrin is involved in ventral extension in *P. pacificus*.

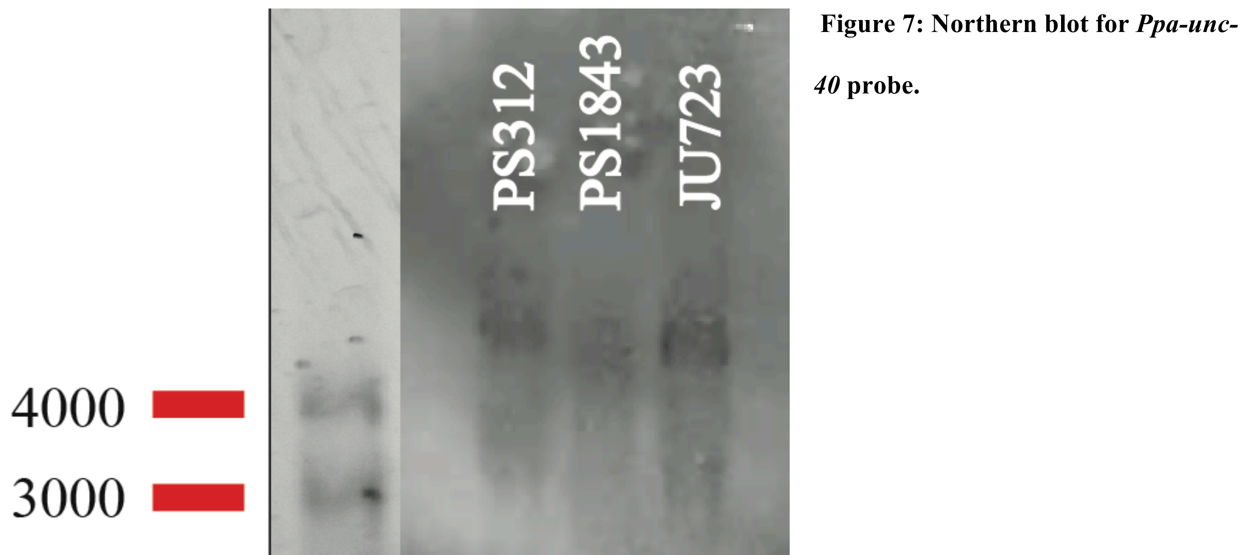


Figure 7: Northern blot for *Ppa-unc-40* probe.

The digoxygenin-labeled probe was also used in RNA *in situ* hybridizations to stain fixed tissues and observe endogenous *Ppa-unc-40* mRNAs. We anticipated staining in DTCs, though the staining is potentially dynamic and dependent upon developmental stage. In whole-mount animals, a very small percentage of DTCs stained in adults (fig. 8). Gonads were dissected out of

the body to increase the permeability of the arm. In dissected animals, the DTC and the rest of the gonadal arm stained (fig. 9) suggesting maternal loading of *unc-40* in oocytes. We are currently working on the conditions for sense controls.

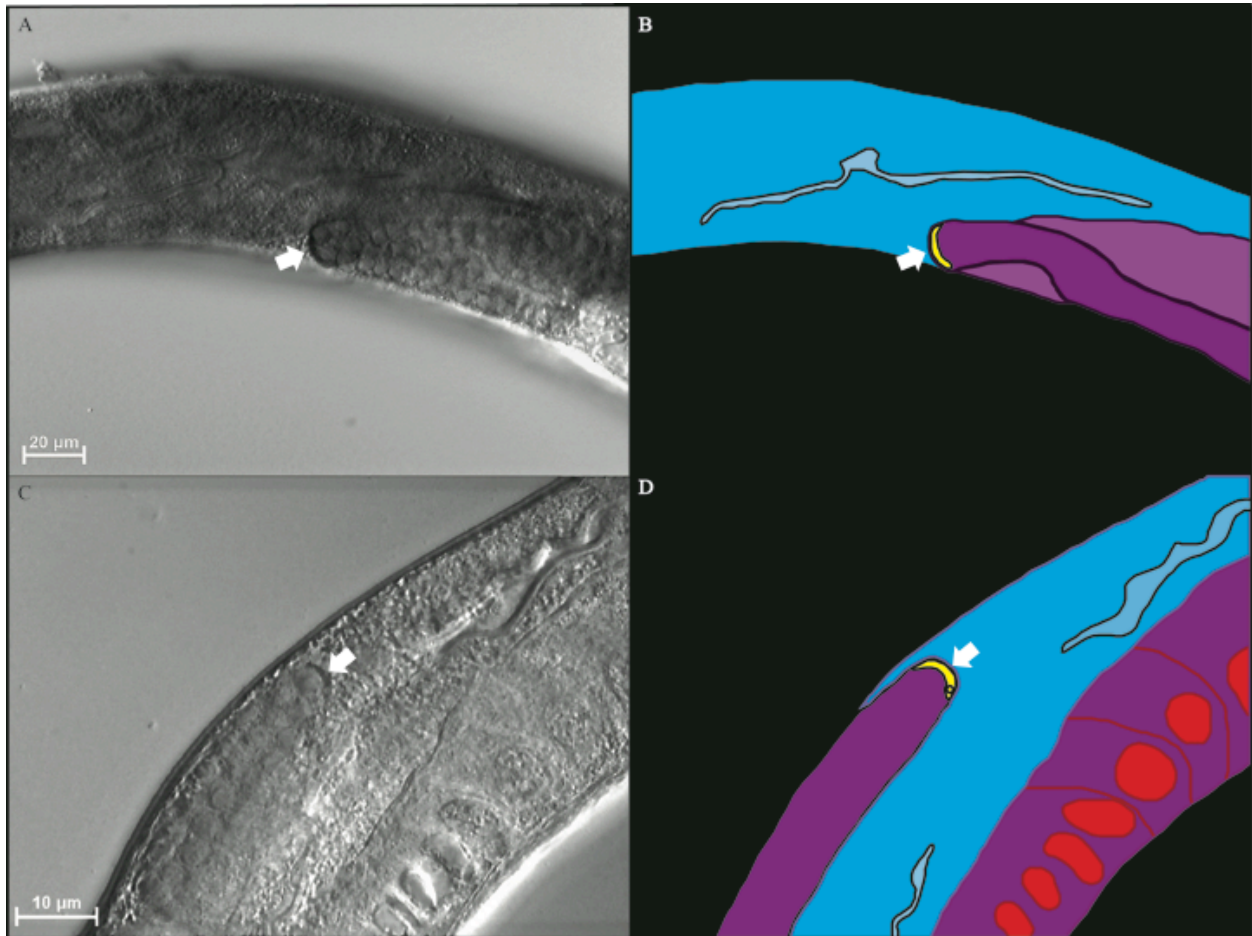


Figure 8: ***Ppa-unc-40*** **RNA** ***in situ*** **hybridization in whole-mount animals. (A & C) Whole-mount** ***P. pacificus*** **strain PS312 showing DTC staining. (B & D) Cartoon representing whole-mount animals. Gonad is shown in red and purple. DTC is shown in yellow. Gut is shown in light blue. Arrow is pointing to location of the DTC. In (D), the circle inside the DTC represents the position of the DTC nucleus.**

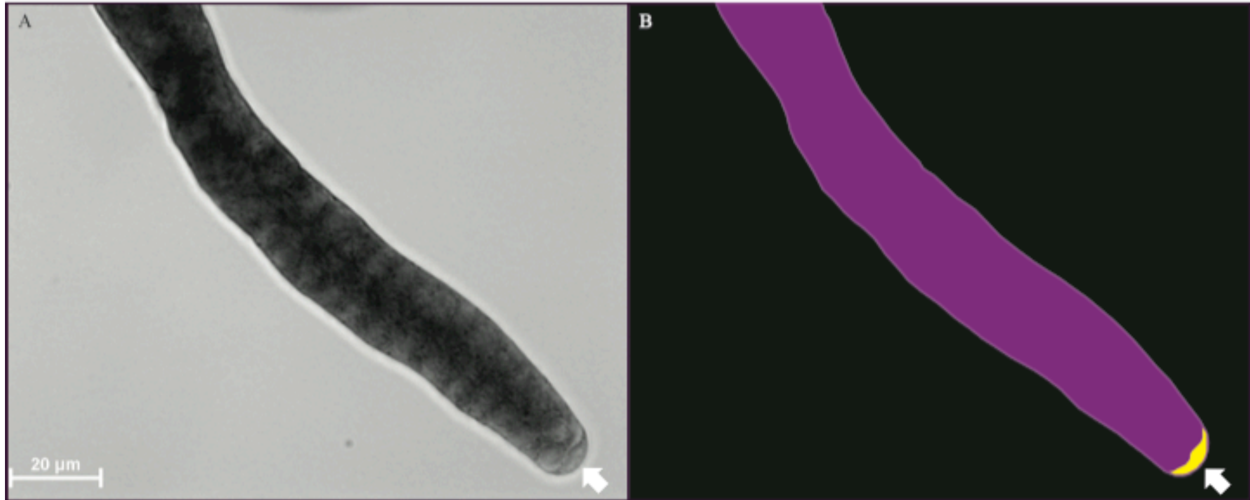
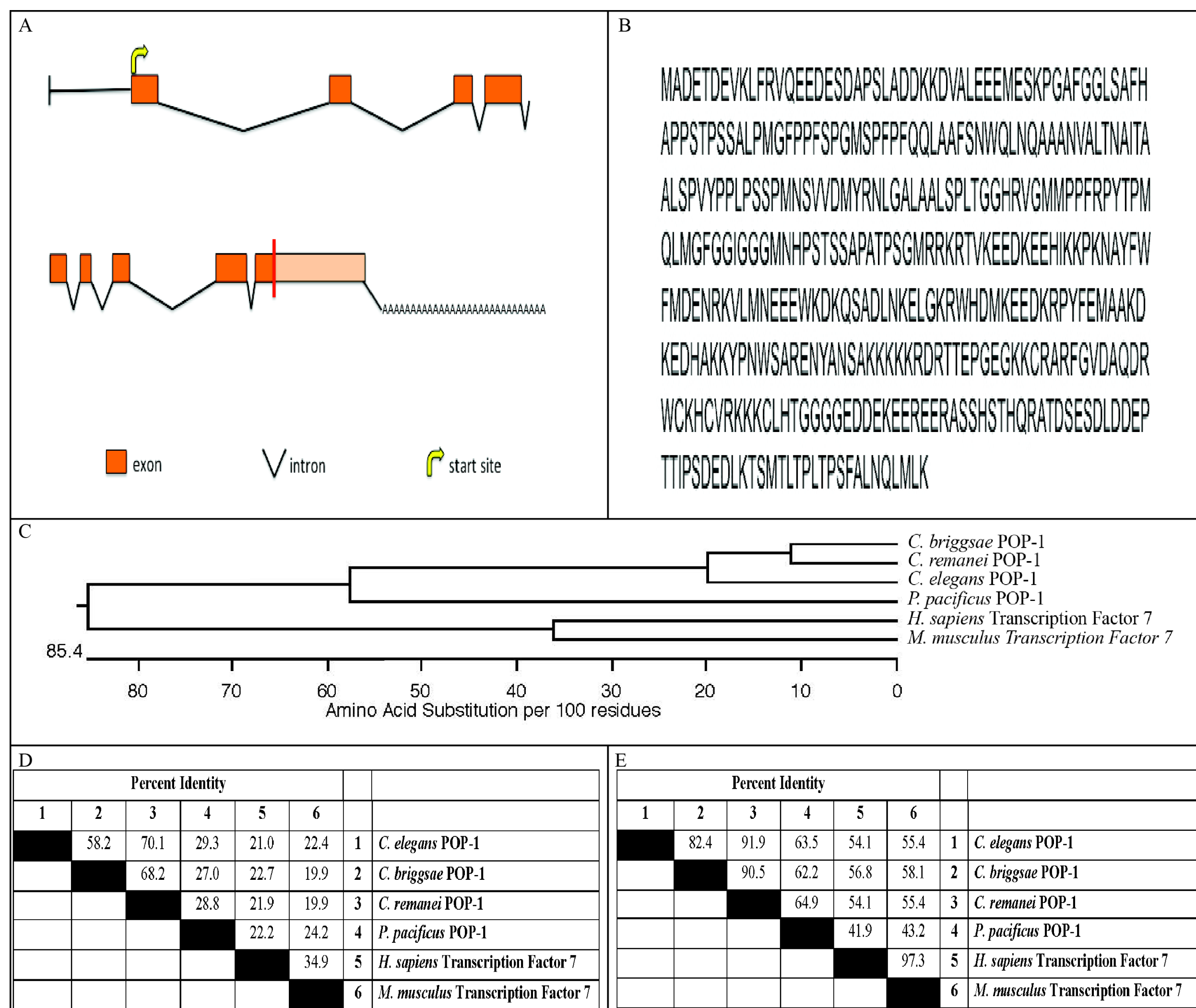


Figure 9: *Ppa-unc-40* RNA *in situ* hybridization in dissected gonads. (A) Gonad dissected from *P. pacificus* strain PS312 showing DTC and gonad staining. (B) Cartoon representing dissected gonad. Gonad is shown in purple. DTC is shown in yellow. Arrow is pointing to the location of the DTC.

Ppa.pop-1 Sequence Analysis

The cDNA sequence for *pop-1* was also confirmed by reverse transcription PCR (fig. 10A). Reverse transcription PCR using the SL1 transplice leader primer yielded a single product. The *pop-1* cDNA sequence is 1748 nucleotides long. The *pop-1* cDNA sequence is found entirely in Contig182.14 based on the genome assembly found on www.pristionchus.org. Like UNC-40, the identity when comparing the whole POP-1 protein between species is unremarkable (fig. 10D). However, when comparing the portion of the sequence associated with the HMG box superfamily, a DNA binding domain, there is 63.5% identity between *P. pacificus* and *C. elegans* (fig. 10E). The high identity and placement within a TCF/LEF-1 phylogenetic tree suggests the sequence we found is *Ppa-pop-1*.



Percent Identity							
1	2	3	4	5	6		
	58.2	70.1	29.3	21.0	22.4	1	*C. elegans* POP-1
		68.2	27.0	22.7	19.9	2	*C. briggsae* POP-1
			28.8	21.9	19.9	3	*C. remanei* POP-1
				22.2	24.2	4	*P. pacificus* POP-1
					34.9	5	*H. sapiens* Transcription Factor 7
						6	*M. musculus* Transcription Factor 7

Percent Identity							
1	2	3	4	5	6		
	82.4	91.9	63.5	54.1	55.4	1	*C. elegans* POP-1
		90.5	62.2	56.8	58.1	2	*C. briggsae* POP-1
			64.9	54.1	55.4	3	*C. remanei* POP-1
				41.9	43.2	4	*P. pacificus* POP-1
					97.3	5	*H. sapiens* Transcription Factor 7
						6	*M. musculus* Transcription Factor 7

Figure 10: *Ppa-pop-1* sequence. (A) Representation of the genomic sequence. The boxes represent exons and the solid lines represent introns with confirmed sequences. The vertical red line represents the position of the stop codon. (B) Protein sequence. (C) POP-1 phylogenetic tree. (D) Overall similarity of POP-1. (E) Similarity of the POP-1 HMG box superfamily.

<u>*Ppa-pop-1* RNA *in situ* Hybridization</u>

A *Ppa-pop-1* digoxygenin labeled probe was also generated from reverse transcribed cDNA and covered almost the entire cDNA sequence for *pop-1* from exon 1 to exon 8. In gonadal arms that were dissected out of the body, the DTC and the rest of the gonadal arm stained (fig. 11) suggesting maternal loading of *pop-1* in the oocytes. We also saw staining in

embryos that were freeze-cracked (see appendix fig. 16). We are currently working on the conditions for sense controls.

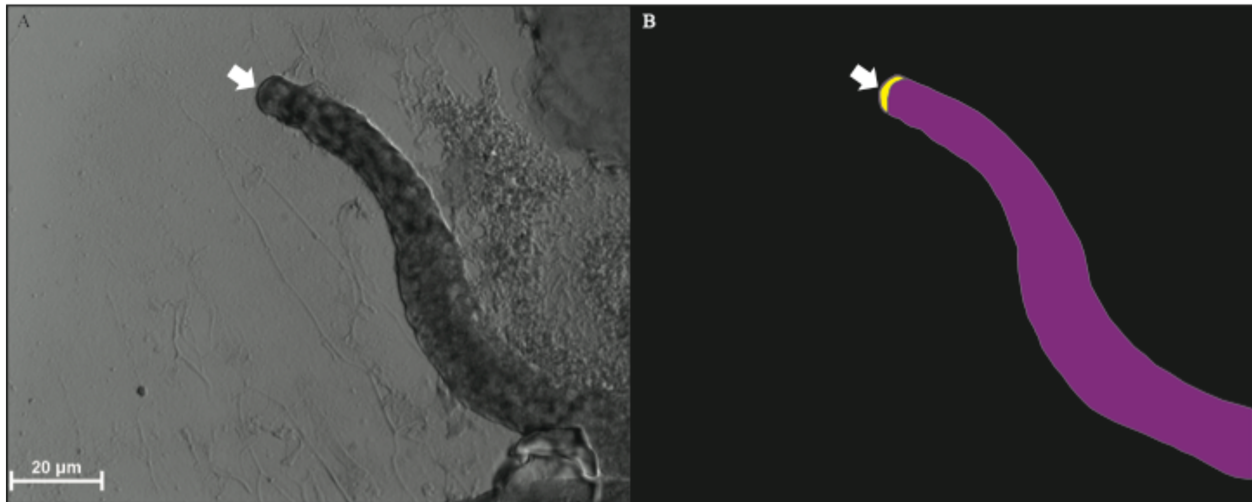


Figure 11: ***Ppa-pop-1*** **RNA** ***in situ*** **hybridization in dissected gonads. (A) Gonad dissected from** ***P. pacificus*** **strain PS312 showing DTC and gonad staining. (B) Cartoon representing dissected gonad. Gonad is shown in purple. DTC is shown in yellow. Arrow is pointing to the location of the DTC.**

Discussion

Our goal with these experiments was to begin to address how the DTCs detect and interpret dorsal/ventral positional information. Based upon data in *C. elegans*, we hypothesized the Netrin cell guidance cue is involved in this process. Based on our model we hypothesized *unc-40*/Netrin receptor was localized to the DTCs in *P. pacificus* to interpret the Netrin guidance cue. Although our protocol did not produce uniform staining in all animals within an experimental treatment, a small number of DTCs showed staining in whole-mount animals. Overall in whole mount animals no noticeable staining was observed in any of the other internal organs of the animals. In this initial protocol, infrequent staining of the DTCs were obtained utilizing only freeze/thaw cycles to create cracks in the cuticle coupled with proteinase K treatment. As we attempted to improve the percentage of DTC staining, we tried to increase the permeability in the animals and began using a freeze-cracking technique on whole mount animals. This resulted in both the DTCs and the rest of the gonadal arm staining in most animals, only a small number of animals showed no staining at all. As a second alternative, we attempted using gonads that were dissected out of the animal with and without freeze cracking. Once again, with dissected gonads we saw staining in both the DTCs and throughout the gonadal arm both with and without freeze cracking. Perhaps *Ppa-unc-40* is a maternally loaded transcript. Occasionally with dissected gonads, we would see a gonadal arm stain and not the DTC, but this was rare. This could have several causes. First, the distal portion of the gonad arm is more susceptible to washing steps. The tip is thinner and more exposed than more proximal portions of the gonad arm making it more permeable to staining and the subsequent washes. Under more stringent conditions, the probe is more easily washed out. Additionally, concerning staining of the distal germ line, maternally loaded transcripts are in higher abundance in the proximal

portion of the gonad. We observed that as hybridization temperatures are increased in our RNA *in situ* protocol, staining progressively ebbed from the distal tip proximally along the arm. Second, this procedure was performed on PS312, a strain of *P. pacificus* in which the arms extend ventrally only ~70% of the time. It's possible these unstained DTCs from dissected gonadal arms were found on gonadal arms that did not extend ventrally.

Given these results and the observation that in completely stained gonadal arms the common cytoplasm is stained, we hypothesize that *unc-40* may be maternally loaded. One experiment that may provide evidence for or against this hypothesis would be to ablate Z2 and Z3 in a population of animals. Z2 and Z3 are the precursor cells that develop the germ line. RNA from this population of animals without a germ line and RNA from a similar group that were not ablated could be used for RT-PCR experiments to see if there is a reduction in *unc-40* signal in the ablated group, providing evidence for a germ line expressed population of *Ppa-unc-40* transcripts.

Previous experiments have shown that UNC-40 is expressed at a constant level in the DTCs throughout their extension (Chan et al. 1996) and UNC-5 is sufficient to induce dorsal extension in *C. elegans* (Su et al. 2000). Perhaps *unc-40* mRNA is present all the time and regulation is at the level of the UNC-40 protein so that the expressed at the appropriate times to induce proper ventral/dorsal migrations. This difference would explain how the Netrin guidance is able to function properly in *P. pacificus* in the absence of UNC-5. In future studies the lab plans to address this hypothesis by studying UNC-40 expression and localization with antibody studies and *unc-40::gfp* translational fusion constructs. The cDNA sequence obtained from this study is being used to order antibodies and to construct PCR fusion constructs to generate the

GFP reporters. The goal of these studies will be to determine the dynamic expression of UNC-40 protein in the DTC throughout development.

Another possibility is the Netrin guidance cue is not the only signal involved in DTC migration. Dorsal/ventral axon migration in *C. elegans* is also controlled by Slit and its receptor SAX-3/Robo. SLT-1/Slit is secreted from the dorsal side of the animal and the SAX-3/Robo receptor is repulsed from SLT-1 to guide developing axons towards the ventral body wall (Quinn et al. 2006). SAX-3/Robo is known to also bind to UNC-40 (Yu et al. 2002) and in the absence of SLT-1 and EVA-1, a SLT-1 receptor, SAX-3/Robo can suppress UNC-40 signaling (Fujisawa et al. 2007). Considering this, it may be possible that the Netrin guidance cue and Slit are acting in parallel to guide DTC migration in *P. pacificus*.

MIG-10 is an outgrowth promoting protein that has been shown to work in both Netrin and Slit signaling pathways (Chang et al. 2006; Quinn et al. 2006). Asymmetric localization of MIG-10 localizes actin polymerization to the site of outgrowth in axons (Quinn et al. 2008). The asymmetric localization of MIG-10 is dependent on Rac (Quinn et al. 2008) and Rac has been shown to be activated in response to UNC-40 signaling (Li et al. 2002; Shekarabi et al. 2002 and 2005). MIG-10 interactions with SLT-1 are not well understood, however, overexpression of MIG-10 enhances the repulsive cue away from SLT-1 (Quinn et al. 2006). Perhaps MIG-10 is involved in enhancing the attractive and repulsive cues of Netrin and Slit in *P. pacificus* DTC migration.

We also looked at *pop-1* localization in *P. pacificus*. We wanted to see if there was a correlation between *pop-1* expression and *unc-40* expression in the DTCs. For instance, is *pop-1* localized to the DTCs at the same time *unc-40* is localized to the DTCs therefore providing evidence that POP-1 is involved in activation of *unc-40* transcription? As with *unc-40* we saw

that both the DTC and the rest of the gonadal arm stained when hybridization was performed on dissected gonads. This result lead us to hypothesize that *pop-1* is maternally loaded as well. This would not be surprising as *pop-1* occurs maternally and the *pop-1(zu189)* mutant only affects maternal expression. In fact, maternal expression of *pop-1* is required for embryogenesis (Lin et al. 1995). Furthermore, asymmetric localization of *pop-1* has been shown to be involved in cell polarity decisions. During development, EMS divides producing blastomeres E and MS. E will develop intestinal cells and MS will develop pharyngeal and body wall muscle cells (Sulston et al. 1983). In *pop-1* mutants, MS will adopt an E like fate (Lin et al. 1995). This is due to improper anterior/posterior fate specification in dividing daughter cells. In wild-type MS and E cells, there is a higher level of POP-1 in the anterior cell compared to the posterior cell. Furthermore, POP-1 is involved in other anterior/posterior decisions such as the AB descendants (Lin et al. 1995). In future studies the lab plans to study the expression and localization of POP-1 with antibody studies and *pop-1::gfp* translational fusion constructs. The cDNA sequence obtained from this study is being used to order antibodies and to construct PCR fusion constructs to generate GFP reporters. The goal of these studies will be to determine if POP-1 is localized to the DTC nucleus and if so, during which phase(s) of migration it is expressed. Our lab would like to determine if there is a correlation between POP-1 localization in the DTC nucleus and UNC-40 localization to the DTC. This will provide evidence for whether a Wnt signal is involved in turning UNC-40 expression on or off.

This study provides the initial observation that UNC-40 is putatively in the DTCs and that the Netrin guidance cue may be involved in gonadogenesis in *Pristionchu*s, Our initial *in situ* results encourage further experimentation. The cDNA sequences for *unc-40* and *pop-1* will allow the lab to test regulation of these genes at the level of the protein. Ultimately, allowing our

lab to contribute to the understanding of how a simple epithelial tube can change shape and further our understanding of the development of organ morphology.

References

Bishopric NH. (2005). Evolution of the Heart from bacteria to man. Annals of the New York Academy of Sciences 1047:13-29.

Blumenthal T. (2012). Trans-splicing and operons in *C. elegans*. Wormbook 20:1-11.

Brenner S. (1974). The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

Chan SS, Zheng H, Su MW, Wilk R, Killeen MT, Hedgecock EM, Culotti JG. (1996). UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 Netrin cues. Cell 87(2):187-195.

Chang C, Adler CE, Krause M, Clark SG, Gertier FB, Tessier-Lavigne M, Bargmann CI. (2006). MIG-10/lamellipodin and AGE-1/PI3K promote axon guidance and outgrowth in response to slit and netrin. Current Biology 16(9):854-862.

Contreras V, Friday AJ, Morrison JK, Hao E, Keiper BD (2011) Cap-independent translation promotes *C. elegans* germ cell apoptosis through Apaf-1/CED-4 in a Caspase-dependent mechanism. PLoS One 6(9):e24444.

Culotti JG, Merz DC. (1998). DCC and netrins. Current Opinion in Cell Biology 10(5):609-613.

Eisenmann DM. (2005). Wnt Signaling. WormBook 25:1-17.

Freeman S. (2005). *Biological Science*. Pearson Education 2nd edition 499-500.

Fujisawa K, Wrana JL, Cullotti JG. (2007). The slit receptor EVA-1 coactivates a SAX3/Robo Mediated guidance signal in *C. elegans*. Science 317(5846):1934-1938.

Guiliano DB, Blaxter ML. (2006). Operon conservation and the evolution of trans-splicing in the Phylum Nematoda. PLOS Genetics 2(11):e198.

Hedgecock EM, Culotti JG, Hall DH. (1990). The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. Neuron 4(1):61-85.

Hillier LW, Coulson A, Murray JI, Bao Z, Sulston JE, Waterston RH. (2005). Genomics in *C. elegans*: so many genes, such a little worm. Genome Research 15(12):1651-1660.

Hubbard EJ, Greenstein D. (2000). The *Caenorhabditis elegans* gonad: a test tube for cell and developmental biology. Developmental Dynamics 218(1):2-22.

Jungblut B, Pires-daSilva A, Sommer RJ. (2001). Formation of the egg-laying system in *Pristionchus pacificus* requires complex interactions between gonadal, mesodermal and epidermal tissues and does not rely on single cell inductions. Development 128(18):3395-3404.

Killeen MT, Sybingco SS. (2008). Netrin, Slit, and Wnt receptors allow axons to choose the axis of migration. Developmental Biology 323(2):143-151.

Lee KZ, Sommer RJ. (2003). Operon structure and trans-splicing in the nematode *Pristionchus pacificus.* Molecular Biology and Evolution 20(12):2097-2103.

Lehmann R. (2001). Cell migration in invertebrates: clues from border and distal tip cells. Current Opinion in Genetics and Development 11(4):457-463.

Li X, Saint-Cyr-Prouix E, Aktories K, Lamarche-Vane N. (2002) Rac1 and Cdc42 but not RhoA or Rho kinase activities are required for neurite outgrowth induced by the Netrin-1 receptor DCC (deleted in colorectal cancer) in N1E-115 neuroblastoma cells. Journal of Biological Chemistry 277(17):15207-15214.

Lin R, Thompson S, Priess JR. (1995). POP-1 encodes an HMG box protein required for the specification of a mesodermal precursor in early *C. elegans* embryos. Cell 83:599-609.

Livesey FJ, Hunt SP. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and Cerebellar development. Molecular and Cellular Neuroscience 8(6):417-429.

Macneil LT, Hardy WR, Pawson T, Wrana JL, Culotti JG. (2009). UNC-129 regulates the balance between UNC-40 dependent and independent UNC-5 signaling pathways. Nature Neuroscience 12(2):150-155.

Mayer WE, Herrmann M, Sommer RJ. (2007). Phylogeny of the nematode genus Pristionchus and implications for biodiversity, biogeography and the evolution of hermaphroditism. BMC Evolutionary Biology July 2;7:104.

Mayer WE, Merrmann M, Sommer RJ. (2009). Molecular phylogeny of beetle associated diplogastrid nematodes suggests host switching rather than nematode-beetle coevolution. BMC Evolutionary Biology Aug 24;9:212.

Olson EN. (2006). Gene regulatory networks in the evolution and development of the heart. Science 313:1922-1927.

Pires-DaSilva A, Sommer RJ. (2003). The evolution of signaling pathways in animal development. Nature Review Genetics 4:39-39.

Quinn CC, Pfeil DS, Chen E, Stovall EL, Harden MV, Gavin MK, Forrester WC, Ryder EF, Soto MC, Wadsworth WG. (2006). UNC-6/netrin and SLT-1/slit guidance cues orient axon outgrowth mediated by MIG-10/RIAM/lamellipodin. Current Biology 16(9):845-853.

Quinn CC, Wadsworth WG. (2008). Axon guidance: asymmetric signaling orients polarized Outgrowth. Trends Cell Biology 18(12):597-603.

Rudel D, Riebesell M, Sommer RJ. (2005). Gonadogenesis in *Pristionchus pacificus* and organ evolution: development, adult morphology and cell-cell interactions in the hermaphrodite gonad. Developmental Biology 277:200-221

Rudel D, Tian H, Sommer RJ. (2008). Wnt signaling in *Pristionchus pacificus* gonadal arm extension and the evolution of organ shape. PNAS 105(31):10826-10831.

Shekarabi M, Kennedy TE. (200). The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. Molecular and Cellular Neuroscience 19(1):1-17.

Shekarabi M, Moore SW, Tritsch NX, Morris SJ, Bouchard JF, Kennedy TE. Deleted in colorectal cancer binding netrin-1 mediates cell substrate adhesion and recruits Cdc42, Rac1, Pak1, and N-WASP into an intracellular signaling complex that promotes growth cone expansion. Journal of Neuroscience 25(12):3132-3141.

Silhankova M, Korswagen HC. (2007). Migration of neuronal cells along the anterior-posterior body axis of *C. elegans*: Wnts are in control. Current Opinion in Genetics and Development 17(4):320-325.

Su M, Merz DC, Killeen MT, Zhou Y, Zheng H, Kramer JM, Hedgecock EM, Culotti JG. (2000). Regulation of the UNC-5 Netrin receptor initiates the first reorientation of migrating distal tip cells in *Caenorhabditis elegans.* Development 127:585–594.

Wadsworth WG. (2002). Moving around in a worm: Netrin UNC-6 and circumferential axon guidance in *C. elegans.* Trends in Neuroscience 25:423–429

Appendix

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ATTGCCAATATGCCTTCTGGTGGTCTTCGTTCATTCTCTAATATTAATGGAGGTCTCT
CTACAGTCAGACAGATTCCAATCGGAAGAGCTCAAGCTCAACCTCGAGTTAATGTA
GCCAATCTGTGCAGxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxTCCGTA
TGTGTCATCGTCGGCAATGTCGGTAGAAGATGAGAAGATGGGATATAAAGAGGATG
ACATGAGTAATCCATCTCATCTCACTCTTCATCAATCAATCTCAACAGAAGAATTGG
ATGAAATGGGAGACGTTGATATCATGATTACAGATCTGCAGGCTCTTCAACAAGAGT
TCGGTGTTAATCCATAGtctcttactctatcgctttctatttgtgcattcttgtgtattgtctgtttctgtttttatctcactaatgatatct
cctctgtactaatgtgttcacttgaccgtttctattaccattccaatataggccctttctcccctcttctcattgataagcgactgtacctttattgaca
ttgacgtgtatcgacgttcttgctttcgacgagaattattactctccattcttgctcaattgaccaatcaataagtatcattgtatatctaagcaatac
ctctttctgtcacattcttctttctgtaaatatattgtcacttcatgttcgtgcttcttcccattcactgtcttttctcaggtatattctgtcaatgctaagc
attaataacacattgtcaaaattgtaataatttattttaaagatcatgacgatgtaaaagtaacaattaacagataaagaaagtcccgttt

Figure 12: *Ppa-unc-40* genomic sequence. UTR regions are in lowercase letters and in a gray font. Introns are in lowercase letters and in a black font. X's represent introns that are not completely sequenced. We are currently working or confirming these introns with reverse transcription PCR. Exons are in uppercase letters and highlighted either yellow or orange to distinguish one exon to the next.

AAATTGTTGAGTATGACTAAATTGGAAATAGTTTACTAGTCTGTCTGATAAGGGGCA
AATAAATGTCCATTATAACTAATATAGGTATTTTCCTGCTCTTCTTATTAACAGTACA
ATGTGAGCAAAGGAAGAGACATCACAGACGATCAATCAGTTATGATGATCGATGGT
TCGGATTTCACTTCGATAATGAACCAATATCGTCAACGTTCCATGGAAAGCGCACTC
TCATACCATGCACATATTCTCTAGTTCGAAAAAGAGAAAGTGTCGTCGAATGGAGAC
GAGACTCTACTCCAATTAACGTTTCCGATAAGAGAATATCAATTCTTGGTAATGGCT
CTCTGTCATTGAATGATGCATCTCGATCTGATGAGGGAGTGTATCAATGTGCTGTCC
ATATTACTGATGATGATGGTTCCACTTGGACTTTCCTCTCCAATAAGGCTGTTATGAG
AGTAAAAAATACTCTAAGATGGAAGGAAGAACCGAAAGATACGACGATTCAAGAA
GGAAAGAGGGGAATACTCAGATGTGAATTGGATGAGAAAAACAAATCGACTATTCG
ATGGATCAAAGACGGGAAAGAGATAACTGAATCAAATCATTTGTTGAAGAATGGAT
ACACAGGAAGTATGGAGATTCGAGGGATGAGAAAGGAGGATGAGGGAGTTTACTC
GTGTATAGCAACTAGAGATAATGAAAGAATTGAAAGTGCACGAGCAAGGATTTCAA
TGAAGAAAGGGAATGAACCAATTGATCTTCATTTTCTCTCTCGTCCGATTTCGAGAA
GAATGAAAGAAGGAGAAGAGACTTTACTTGAATGTATTCCATCGGGAGAAGGAGAA
TTACGAGTGGAATGGACTAAAGATGGACGGAAAATTGATGATCTCAAAGGAATTCA
GAGGATGGGGTCGTCTCTCTATATTTCTTCACTTCAATTTGATCATTCTGGAATGTAT
ACGTGTAGAGTGAATGATTCGACTGATTCAATCGAAGCATCAGCTACTCTCACTCTT
ATTGCTCCTCCTCATATAACTTCTCGCCCTCAGGATATTGTTGCTGTGGAGACAACTG
ATGTTGAAATGGAATGCTATTCATCTGGACGCCCGCCTCCAATTCTTTCCTGGTTTAA
AAATGGAGAAATGCTTGTTCCCAGTGAATACTTCGTTATTGAATCGAACAAACTGAG
GATTCTCGGCGTCGTCAAAACTGATCAAGGGATATACGAGTGTTTTGTCGAAAACGA
AGGAGGGACTGCTCATGCAGCAGCTCAATTGTTGGTGGATAATACTGATTCTTCATT
GATCGCCGCCCATTCTGGTACTCCAGTGAAAGTATCTGCCCCTCTTGGATTGGGAGT
TACTGTGATTGGAAGTAGAGAAGCTACTCTCATTTGGGACCCTCCTATTCAAAAGAA
TGGAAATATACTTCATTACCATTTATATTACAAGGAGGAAGGAAGTGATCGTGAGCG
AGTAGTTAATTCAACGGATCGAGTTGGTCGATTACGTGATCTTCATCCATCGACAAG
TTATCTCATTAGAGTAGCAGCTGAGAATGAAGCTGGAATAGGAGATATATCCAATCC
CATTACACTCACTACCAAGGAAGAACAAGAAGTCCCTGGACGAGTTGTGAATTTGA
GTGCAATTGGAATTGGATCAGAAACGATTCAAGTTGAATGGAGAAAACCATCGAAT
GGAGGAGCAATTCCCCTATTCTATCGATTATTCTATGTTAGAAGTGATAGAAAACTA
ATGGAGAAGGAGACACAAATAAATGTGCCATCAACTGCCTACACTATGCATGGAAT
GGATAAATACACATCATATGATATTCGAGTAGAAGCAGAAGGAGAGAAGGGAGCTG
GTCTCTCATCGAATACCGTAACCGTACGAACTCTATCTGATCTCCCATCCATGGCTCC
CATTGATATAACTGCAAGATCTATTTCGACTACTTCGATTAAAGTGGCGTGGAAAGT
GGATGAAAGTCAATCGAATGGACCAATCACTGGAGCGAGAATGAAGATGAAGACTA
AAGCGCGAGGAAGCAAAGCATCGAATATTGTTATGGATTCTTCTCGATTGGAAGAA
ATTGTAGAAGGATTAGAGGCTGGAACTGCATATCAACTGAGAATTGCACTTGTTAAT
CAGAATGGACAGGTCCTTATTCTGATTGGATATCAGTGGATACTCCATTAACTGAG
AAGGATGAATCTGTATTGGGAGCTCCACGAGAGTTAACAGCTCGTGCCACGAGTGA
TTCTATCTCTCTTCGATGGATTCCCCCTGCTGATGAAAGTGTACTCGTCAGAGGATAT
CAAATTGGATGGGGTCTTAATGTTCCTGATGTCGATTCATCGCGAGTCGATGGAGAT
ATTCATGAGTTCAAGATCAGTGGATTGAAACCATCGAGGGATTACGTTATCTCAGTG
AGGGCATTTAATAGACAAGGAAGTGGATTTCCCATCTATGAGACTGTCAGAACATCT
TCAACTGGAGCATCTATGATTGGAGGAGAGGGAATCGATGGAAAGGAAGTGAAAT

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CGATTAGATTGGGCTGATAATCCATCTCTATTTAATATTGTATACAATGTTAAATATA
TGAGCAAGATTGACTCTGGTCAAGCCCGTTTCCTCAATACCTCCGAATCATGGATCG
TAGTGGATAAATTGAAGCCAGGGACAGAATACGAATTGGCAGTTCGTTGTATGGTA
CCCCCTTCATCTCTTTCCCCATGGAGTATGACTGTATCACAAGTGACTCTTCCTACTG
CTCCCTCTTCTTCTCCCAGGGATGTAACAGTTCTTCCTGCTGCATCAGGAGATCCCCA
TTCTGTTACTGTCAATTGGCAACCACCCAAATATGCTAATGGAGAATTGACTGAGTA
CATAGTGTATTATACTGATCGTGGATCAGCTCGTGAAGAAGAATGGATTTCTATTCG
AGTTGCACCTGATAGATTATCTGTTCCCATTGATAATCTACTTCCAAAAGCTACTTAT
TACTTCAGAATACAAGCAAAGAATGAGAAAGGAATGGGTCCGATGTCTCCTAATCA
ATCATATTCCCCTGGTTCCGCTGGTTTCGTTCATCCCACTGGAAACTCTGTAAAGACG
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GAGAAGATACATATGCGTGTGTTATCGGTGGTCGTCGTATTCCATCTCTTCCATACG
ATAATGAATCATCTATTGATTCAACTCGTCCTCTTCTCTCATCTCGAATAACTCATAA
ATCTCATCCTCATATGGTTCAAGTCGCTCCACCTGCCCCTGGATCATCTACTGGAGAT
AGTGAGAGGAGTGGAACTTTGTCTCGAAGTTATCAACATTCAACAACTTCTCTTGAT
CAGCGACAACGTACTCCCCATATGGTATACGGAGGGCATGGAAGGGGAATGGGTAA
GATCGATATAAGTGAATCTCCATATGGATCATGGATGGGTCTACCATCGGGTACTCC
TCCTCCTCCTAATCTTGCCCCTCCTCAACCACCTATTGTTACTGCTGATGGGTATCGA
TCCATTAGAGATGCTTCGAATATTGCCAATATGCCTTCTGGTGGTCTTCGTTCATTCT
CTAATATTAATGGAGGTCTCTCTACAGTCAGACAGATTCCAATCGGAAGAGCTCAAG
CTCAACCTCGAGTTAATGTAGCCAATCTGTGCAGTCCGTATGTGTCATCGTCGGCAA
TGTCGGTAGAAGATGAGAAGATGGGATATAAAGAGGATGACATGAGTAATCCATCT
CATCTCACTCTTCATCAATCAATCTCAACAGAAGAATTGGATGAAATGGGAGACGTT
GATATCATGATTACAGATCTGCAGGCTCTTCAACAAGAGTTCGGTGTTAATCCATAG

Figure 13: *Ppa-unc-40* cDNA sequence. Exons are highlighted either yellow or orange to distinguish one exon to the next.

ctcataccgagagagagacctcggcctctcctctaagtcttctcagattacgaccattaccgtataccgccagtcattaaatcccccaacctcc
aatcttactctgcttttaccaactgtctctctctatcgctttgatatcagtctatcaatctcctcaattgcatgcttcctaccacctcccttcttcttcctc
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ATGAGAATCGGAAAGTGTTGATGAATGAGGAGGAATGGAAGGACAAGCAAAGCGC
CGATCTGAATAAGgtacgattgattgacaatggggtactgtagaagatacggtagtggtcaatctaatcaattatcgatcgctcttc
agGAGTTGGGCAAGCGATGGCACGATATGAAGGAGGAGGATAAGAGGCCGTACTTC
GAAATGGCTGCGgtgagtacggtagattactgtaggattgtatcgattacccatttttcatccatttgactccatccgttagtgtccttt
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AGTATCCCAATTGGTCAGCAAGAGAGAATTATGCGAATAGCGCCAAGAAGAAGAAG
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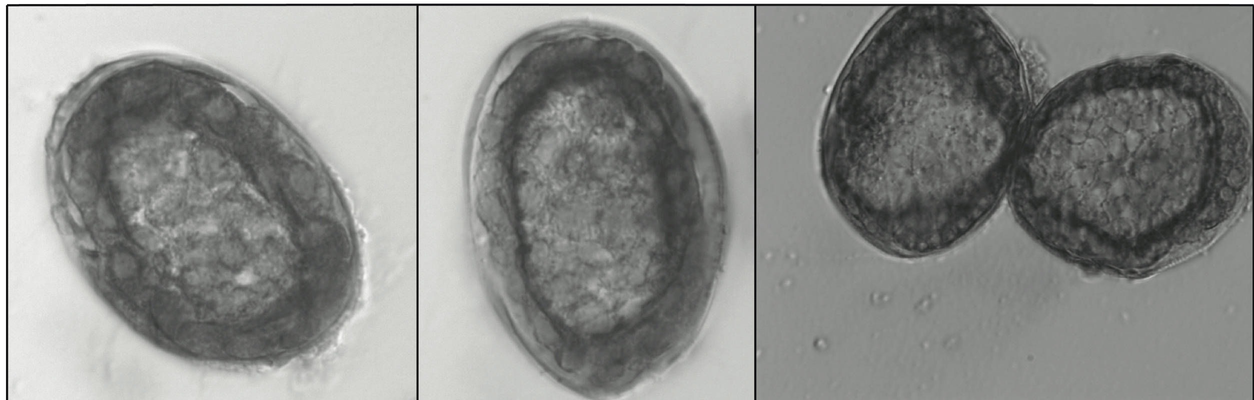
AGCACTGTGTGAGGAAGAAGAAGTGTCTTCATACGGGAGGAGGAGGAGAAGATGA
TGAAAAGGAAGAAAGAGAAGAAAGAGCTAGTAGTCATTCTACACATCAGAGGGCT
ACCGATTCAGgtcagtaaaactgatatggtagatcaatcaatcaataatgaatttgagAATCAGATCTTGACGATG
AGCCCACGACGATTCCATCAGATGAAGATCTGAAGACATCAATGACATTGACTCCTT
TGACACCATCGTTCGCATTGAATCAATTAATGCTTAAATAGaatgaatgaattgagtagtagctaata
cggggcgaatagtgcagtgtagtatccactctaatctttgtcgttgtcttgtatcttcccattgtccctttctgtatctaaccccctgatagatgaat
gcttcttagctatacatcttccattcatgtcccattcagtctctcccttgtatacctcatttcttcttttccgagtaggtctccatttcattctcccagttc
acataatctcattagcccagtctgatggaatagttcaactagagattatcccttcgttccttcaatcgggttcaattattttttggtttgtttgatcatc
acttggtatcattctactaatcgcatactctcaatgagtctcttcttctctctttcacataatagtcagccattcggtccgtctcttgcccagtatgcc

ccctcccttctctctaatcgaatctgtttgtaactataatactttgttaagagtcaatgtaatcgtatttgagtgtgtaacttcgttcatcgtgaatatat
cacttcttcttc

Figure 14: ***Ppa-pop-1*** **genomic sequence. UTR regions are in lowercase letters and in a gray font. Introns are in lowercase letters and in a black font. Exons are in uppercase letters and highlighted either yellow or orange to distinguish one exon to the next.**

AGTATCGTTCCCGTTCCATTTGGAATGGCAGACGAGACAGATGAAGTCAAGTTATTT
CGAGTACAAGAAGAAGATGAAAGTGATGCCCCTTCCCTTGCTGATGACAAGAAAGA
TGTGGCATTGGAAGAAGAAATGGAATCAAAACCAGGAGCATTCGGTGGTCTGAGTG
CGTTCCATGCTCCTCCATCAACTCCATCCTCTGCTCTTCCAATGGGATTTCCACCATT
CTCACCAGGAATGAGTCCATTCCCATTCCAACAATTGGCCGCATTCTCCAATTGGCA
ACTGAATCAAGCTGCAGCGAATGTGGCTCTTACGAATGCGATTACTGCTGCTCTTTC
CCCCGTATATCCACCTCTTCCCTCCTCTCCAATGAACTCTGTTGTTGATATGTACAGG
AATCTGGGAGCGCTCGCAGCCCTGTCTCCTCTTACGGGTGGTCACCGAGTGGGAATG
ATGCCTCCATTCCGACCATACACTCCAATGCAACTAATGGGATTCGGAGGTATTGGA
GGAGGAATGAATCATCCTTCCACTTCCTCTGCCCCTGCTACTCCATCTGGTATGAGA
AGGAAGAGAACTGTCAAGGAAGAAGATAAAGAAGAACATATCAAGAAGCCGAAGA
ACGCGTACTTCTGGTTCATGGATGAGAATCGGAAAGTGTTGATGAATGAGGAGGAA
TGGAAGGACAAGCAAAGCGCCGATCTGAATAAGGAGTTGGGCAAGCGATGGCACG
ATATGAAGGAGGAGGATAAGAGGCCGTACTTCGAAATGGCTGCGAAGGACAAGGA
AGACCACGCTAAGAAGTATCCCAATTGGTCAGCAAGAGAGAATTATGCGAATAGCG
CCAAGAAGAAGAAGAAGAGAGATCGAACAACTGAGCCAGGAGAGGGTAAGAAGTG
TCGTGCTCGATTCGGAGTGGATGCACAGGATAGATGGTGTAAGCACTGTGTGAGGA
AGAAGAAGTGTCTTCATACGGGAGGAGGAGGAGAAGATGATGAAAAGGAAGAAAG
AGAAGAAAGAGCTAGTAGTCATTCTACACATCAGAGGGCTACCGATTCAGAATCAG
ATCTTGACGATGAGCCCACGACGATTCCATCAGATGAAGATCTGAAGACATCAATG
ACATTGACTCCTTTGACACCATCGTTCGCATTGAATCAATTAATGCTTAAATAG

Figure 15: ***Ppa-pop-1*** **cDNA sequence. Exons are highlighted either yellow or orange to distinguish one exon to the next.**



Supplemental Figure 16: ***Ppa-pop-1*** **RNA in situ hybridization in freeze-cracked embryos.**

TCGCATCGGCATATTACTTCTCTCAATTCTCCATGCGCCAACCTGATCATGATCCCTG
CCACGATAATTCCGGTCGACCTGTGCGATGTGTGCCTGAATTCATCAATGCCGCATT
CGGAAAGCCTGTCGTAGCGTCGGATACATGTGGAATGAAGGGAGCAACCAAgtaaacctt
tgataaattctagaaatatcttcctcagtacacaccagtatcttgtctattgcatttactcaatgagctgtactcttgttgactgtgtttttatagcgct
tcagatttcaagttcacgtgtgcatgttaacagtattgactatgcgttcattcaaagctccctattctttatggcgctttttattatgccctaccctttta
gttattatcacagttgacctctatcttctttgatattcaaaattgccgaagcagttcctcctcaaacctctttgcacgcatctcttcactgtttgatgtc
taattctgcccctctttgttccactcactgacacctctctctgtttcttagttggacaactccatttagcctatctcgaccttctttgaaggaaagagg
gagagagaaaagaccgtgtgctagattacatccactttatcactcgttccccttatgagtccctccgctgtgcagaaatgagttatgacgatta
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TCCGTGAGGAATGTTCAACTTGCGATGCCACTCGACCTCATCTCTCCCATCCCGCGT
CCTACCTCACTGATCTGAACAATCCCCAGAACATGACCTGTTGGGTGTCGGAACCTT
CGATGAGCTATCCACGAAATGTCTCTCTTACTCTGTCCCTCGGAAAGAAGTTTGAGC
TGACATACGTCAGTATGCAGTTTTGCAATCGTCTTCCCGATTCGATGGCCCTCTACAA
ATCGGCTGATCATGGTCGAACTTGGTCGCCATTCCAATTCTATTCCACGCAATGCGA
GCAGATGTATGGAAGACGACCGGACGTCAAGATCGAGAAGCACAACGAACAGgtatca
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CATACTCTGCAAATGCGTGTGTCGgttagtgaacttcatctatctcatcttctcaatcccggttcactcggataaatggag
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CCGCTTCGATTCTGAACTGTTCAAAATGAGTGGGGATCGTTCTGGAGGAGTTTGCAT
CAATTGTCGACACAATACGGCTGGAAGGAACTGCCATCTATGCAAGCCTGGTTTCTT
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cctcatctgtgtatggttatgaggatagacgagcacaggtgtttcctgcctcattcattggctcattcgaaaacacgagcagattgaaataagat
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aaagtcgcaactgttcatgtttcgccactcgatgagaaacgagaagacgagtggagaagtcgacaaattgccccctggggctaagattgat
cgagcaatgaggaaagatgtaacctggcgccttcttgaacactcgacacgaattacaacttgagaaggggcgaggagtgaggggactaat
ctagttatccaatacatccgagactcgtcaatagcatgaatgggggatgcatgattgaaattgagttgtattgaaaagGAAAATGTGT
TCAAATGAAAATATTTCAGAATGTAATTGCCACCCCGTCGGATCAATTGGGTCATCG
TGCAATCAGACCTCAGGACAGTGTTTGTGCAAGCCCGGAGTTACCGGACTCATTTGT
AACAAATGCGCAAAGGGATTCGTTCAGTCTCGATCGACCACCACACCTTGCATTCgtg
agttaattgtatgatttaactgaacgataggcgaatagggcaaaatttattctgataaaaagaaattcttcactatcgtggtgttagaacagcaat
agccagagggttatctctcagaagtatgttcgtcaaagctagcaggataatccaccattgagatacagactaatctctctcgattcccattaact
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cttacacagttaaaccacgatagagttgacatggaaagaaagggatccaattttaattcaaaattttaagGTGTCGCTCCTCTTG
ATGTTCCCGTCGCGCCTACTGgtcagtttacattcacacacttcaatcatttactcaatatttgaaacatctagaaatgtttatc
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ATAAATATGGATTTAAGCCCGTTCATGCCCCAAGTGCCGCTCTTCGCCCAAGAGACT
GAACCAGAAAAAATTCTGCAAGCGAGACTACGGTATgtttgatgaaccggcttccacgaggacgaaaa
aaagaaaaataaataaattttcagCCTTGCAAGTACACGTCGTCGGTCGGGAAATTCTTGACAATG
GATGGGCCCGTTACACTGTCGTCGTTGAGTCAGTGATGAAGAAGGCGAgtctataccttatata
ccttgacccaatctgcacatatttgagagggatcgccagatcattgaccagagtgtggacatagaaattcatatattttttttacatttttgatagtc
tatttcggcctagggtcgctggtagtcgattctcgcgcgatactcctcatattctctttgtctcaatctcagtattcctcacattgcagGGCAT
TCGTGGTCGTCGTGGTCAGATGGATATGTGGCAGGAGCCACATGCCCTTTCATGCAA
GTGTCCAAGAGTCAAGGTTGGAAGGAGATATCTCCTACTTGgtacgaatatcttccacatatcacattt
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gattgtgcgaggaaatgatcgagacaggaaattagactatcattgagaatgcattcgcttgaccggaaattgctagattatgcatttccctaaa
gtccctaaagtttaccattgaggcgccgaaaaatatggacgatatgaaaaatgtgaaaagtgtgtgaacatattattgcagGAAAGGA

CGATCCTATTGACCGCGAGCATCCCGGTCTCGTGCTGAATGCGAAGAGTCTGATGAT
CGAATGGACGAAGACGTAATGGACAAGgtacaacatatttttgatgttttcgttgattatagtaatgactgatttcc
cagGTTCTTCGCTTCTCAGAGAAGGACCGAGCTGGCCTTT

Figure 17: *Ppa-unc-6* genomic sequence. Exons are highlighted either yellow or orange to distinguish one exon to the next. This sequence is currently not complete and we are working on confirming the remainder of the sequence.

TCGCATCGGCATATTACTTCTCTCAATTCTCCATGCGCCAACCTGATCATGATCCCTG
CCACGATAATTCCGGTCGACCTGTGCGATGTGTGCCTGAATTCATCAATGCCGCATT
CGGAAAGCCTGTCGTAGCGTCGGATACATGTGGAATGAAGGGAGCAACCAAATTCT
GCTCAACGAAAGAAGGCGCGGACGGAGTAATCCGTGAGGAATGTTCAACTTGCGAT
GCCACTCGACCTCATCTCTCCCATCCCGCGTCCTACCTCACTGATCTGAACAATCCCC
AGAACATGACCTGTTGGGTGTCGGAACCTTCGATGAGCTATCCACGAAATGTCTCTC
TTACTCTGTCCCTCGGAAAGAAGTTTGAGCTGACATACGTCAGTATGCAGTTTTGCA
ATCGTCTTCCCGATTCGATGGCCCTCTACAAATCGGCTGATCATGGTCGAACTTGGT
CGCCATTCCAATTCTATTCCACGCAATGCGAGCAGATGTATGGAAGACGACCGGAC
GTCAAGATCGAGAAGCACAACGAACAGGAAGCCCGCTGCACCTCTTCGCACACGCT
CGGTCTGGCTGGTAATCGGGTGGCTTTTCCCTTTCTCGAGGATCGTCCATCCGCCATA
CACTTTGAACAGTCGCCCGTCCTGCAAGATTGGGTTACCGCAACGGATATTCGAGTG
GTCTTCTCGCGACTGAGTCCCGACCAGGCGGAGCTGTATGGACTAACGAACGACGTT
GGCGATGCGCTAGCAGCAGCCAACATGACTGATGAGAGCGATGGGAAGGAGAGAT
ACTTCTATTCAATGGGCGAACTTGCTGTGGGCGGTAGATGCAAGTGCAATGGACACG
CCGCCAGATGCATTTATGATAAGAATGGACAGATGACATGCGACTGCAAGCACAAT
ACGGCAGGAACGGATTGTGAGAGATGCAAACCATTTCATTACGATCGACCATGGGC
AAGAGCCACTGCATACTCTGCAAATGCGTGTGTCGCCTGTAATTGTAATCTGCACGC
GAAGAGATGCCGCTTCGATTCTGAACTGTTCAAAATGAGTGGGGATCGTTCTGGAGG
AGTTTGCATCAATTGTCGACACAATACGGCTGGAAGGAACTGCCATCTATGCAAGCC
TGGTTTCTTCCGTGACCCCACTAAGCCAATCACACACAGAAAAGTGTGTACAGGAAA
ATGTGTTCAAATGAAAATATTTCAGAATGTAATTGCCACCCCGTCGGATCAATTGGG
TCATCGTGCAATCAGACCTCAGGACAGTGTTTGTGCAAGCCCGGAGTTACCGGACTC
ATTTGTAACAAATGCGCAAAGGGATTCGTTCAGTCTCGATCGACCACCACACCTTGC
ATTCGTGTCGCTCCTCTTGATGTTCCCGTCGCGCCTACTGCCATCAGGGACCTGCCAA
ATAATAAATATGGATTTAAGCCCGTTCATGCCCCAAGTGCCGCTCTTCGCCCAAGAG
ACTGAACCAGAAAAAATTCTGCAAGCGAGACTACGGTATCCTTGCAAGTACACGTC
GTCGGTCGGGAAATTCTTGACAATGGATGGGCCCGTTACACTGTCGTCGTTGAGTCA
GTGATGAAGAAGGCGAGGCATTCGTGGTCGTCGTGGTCAGATGGATATGTGGCAGG
AGCCACATGCCCTTTCATGCAAGTGTCCAAGAGTCAAGGTTGGAAGGAGATATCTCC
TACTTGGAAAGGACGATCCTATTGACCGCGAGCATCCCGGTCTCGTGCTGAATGCGA
AGAGTCTGATGATCGAATGGACGAAGACGTAATGGACAAGGTTCTTCGCTTCTCA
GAGAAGGACCGAGCTGGCCTTT

Figure 18: *Ppa-unc-6* cDNA sequence. Introns are in lowercase letters. Exons are in uppercase letters and highlighted either yellow or orange to distinguish one exon to the next. This sequence is currently not complete and we are working on confirming the remainder of the sequence.

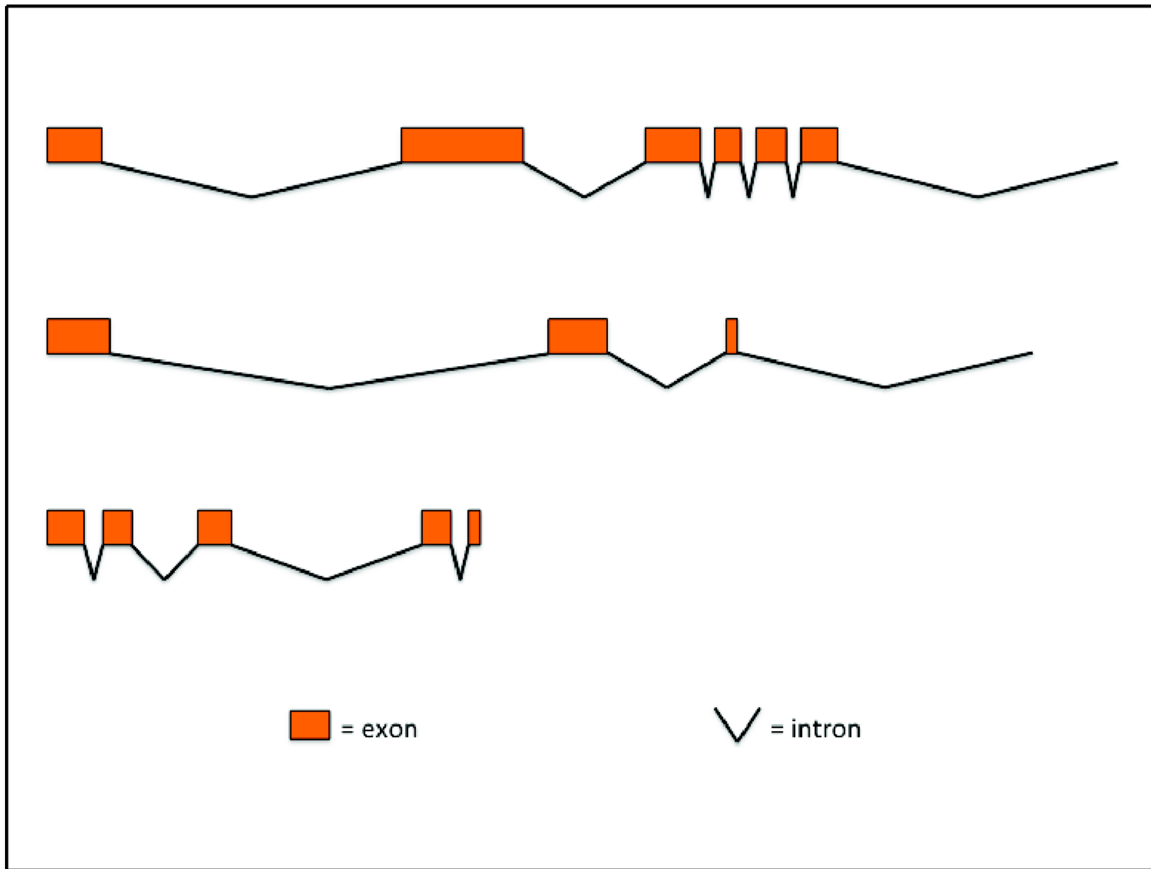

Figure 19: ***Ppa-unc-6*** **sequence. The boxes represent exons and the solid lines represent introns. This sequence is currently not complete and we are working on confirming the remainder of the sequence.**