EVOLUTION OF THE WNT SIGNAL TRANSDUCTION PATHWAY IN *C. briggsae* VULVAL DEVELOPMENT

By

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Dedicated to Appa, Amma, Maya and Baba perippa

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ABSTRACT

Vulval development in *C. elegans* serves as powerful paradigm to understand the interplay of diverse signal transduction pathways during organogenesis. Previous studies have demostrated that the canonical Wnt signaling pathway plays a pivotal role in the development of the vulva in *C. elegans* and helps in establishing the 20-10-20 vulval induction pattern of the vulval precursor cells (VPCs). The main focus of my masters research project was to get an understanding of how this vulval induction pattern, established in response to Wnt signaling has evolved in other closely nematode species, particularly *C. briggsae*. We find that the Wnt signaling pathway has evolved to positively as well as negatively regulate the competence of VPCs in *C. briggsae*. We demonstrate that while mutations in *pry-1*/Axin in *C. elegans* result in Multivulva (Muv) phenotype, mutations in the *C. briggsae pry-1* gene give rise to a novel Multivulva-Vulvaless (Muv-Vul) phenotype. This phenotype is characterized by VPCs anterior to P6.p frequently adopting induced cell fates while those posterior to P6.p frequently adopt a non-induced fate. Furthermore, we also show that the functioning of the Wnt signaling pathway in *C. briggsae* is dependent upon the activity of key regulators of the Wnt pathway such as the TCF/LEF-1 family member *pop-1*, the β-catenin *bar-1* and the hox gene *lin-39*. Taken together, the findings from this study show that while a conserved canonical Wnt pathway confers competence on VPCs in both *C. elegans* and *C. briggsae*, the final outcome nonetheless seems to have diversified.

CHAPTER I: Introduction

1.1 Background

The adage says that truth is indeed stranger than fiction. The saying couldn't be truer especially when it concerns matters of life. The earth is home to millions of living organisms, each one as different as can be from the other. What is strange and yet fascinating about life is that underlying all the diversity and disparity, is a unifying theme that binds all life forms as one. Whether we discuss a single-celled microbe or the aggregate of more than a billion cells that constitute the human body, all organisms on earth without a single exception are generated from cell divisions arising out of a single cell. The information pertaining to the fate and precise patterning of cells, formation of anatomical complexities of tissues and organs are all engraved in long double-stranded helical molecules known as the DNA. This information is then relayed across the vast array of cell populations through channels of communication known as signal transduction pathways.

Signal transduction pathways act as the effectors of cell fate, cell division and body patterning of organisms during development. Research has shown that the functioning of key signal transduction pathways is conserved right from hydra to humans (Cadigan, 1997). Thus, scientists have leveraged this phenomenon to get a better insight into the functioning of complex gene regulatory networks in humans, by studying the role of key

signal transduction pathways during the development of certain organisms with simplified anatomical and genetic characteristics known today as model organisms.

A model organism is essentially a species that is widely studied in order to obtain a deeper understanding of a particular biological process, with the hope that the findings generated in that organism would shed light on the functioning of similar biological processes in other organisms as well, including humans. Today, a wide variety of model organisms are being used in various types of biological research. The nature of these model organisms range from life forms with relatively less complex genomes such as the bacterium (*Escherichia coli*), budding yeast (*Saccharomyces cervisiae*), to organisms with more complex genomes such as the fruit fly (*Drosophila melanogaster*), Zebra fish (*Danio rerio*) and the Mouse (*Mus musculus*) etc.(Landsverk and Epstein, 2005).

Each model organism has its own unique set of advantages and disadvantages. The selection of an appropriate model organism largely depends upon the kind of research question being addressed. However, all model organisms share certain key characteristics that make them particularly preferred for biological research in comparison with most other organisms. These characteristics include the ease of availability of the organism, low cost of maintenance in laboratory conditions, rapid development coupled with a relatively short life cycle and amenability to genetic manipulations (Guarente and Kenyon, 2000).

Interestingly, some of the discoveries made in model organisms have hugely helped researchers to get a deeper insight into some of the fundamental concepts in biology. For example, studies conducted on the banding patterns of the giant chromosomes found in *Drosophila's* salivary glands have helped researchers get a better understanding of chromatin structure and transcriptionally active chromatin regions (Gilbert, 2008). In recent times, model organisms are being widely employed to probe into the potential genetic causes underlying variety of human diseases such as cancer, Huntingtons disease, Alzheimers disease etc., and in the development of effective therapeutic measures (Siddiqui et al., 2008), (Saito and van den Heuvel, 2002), (Link, 2005), (Link, 2006), (Pinkston et al., 2006). The success of this strategy is mainly due to the common descent of all living organisms, and the conservation of key biochemical and developmental pathways over the course of evolution (Wagner, 2000), (Jenner and Wills, 2007).

1.2 *Caeonorhabditis elegans*, the nematode model

The comparison of the development of just a handful of model organisms has offered a powerful insight into the molecular mechanisms governing life itself. Over the past two decades the small soil nematode *C. elegans* has emerged as a powerful tool in the field of evolutionary developmental biology (Jenner and Wills, 2007), (Blaxter, 1998), (Rudel and Sommer, 2003). This nematode model system has been used by researchers seeking to get a deeper insight into some of the profound questions raised in developmental research. For example, how do some of the fundamental developmental processes like

cell division, cell fate specification and body patterning vary among different species in the animal kingdom? And how have these processes evolved? Is it possible for us to get an understanding into the direction of evolutionary change governing these developmental processes? (Sommer, 2005). Just as the old saying goes "A journey of a thousand miles begins with a single step", before we can start answering some of those broad questions raised above, it is vital that we learn something about *C. elegans* itself.

One of the features of *C. elegans* that makes it an attractive model system is the fact that it is a multicellular organism whose development can be studied in considerable detail. Some of the other important features of *C. elegans* that has contributed to its use as a model organism include transparency of the body, low cost of maintenance and a short life cycle (3 days). *C. elegans* also displays a remarkable ability to survive under stressful environmental conditions (such as lack of nutrient availability for example). This ability to survive under difficult environmental conditions contributes to the organism's evolutionary success (Brenner, 1974). Moreover, strains can be frozen and preserved in liquid nitrogen.

Another key feature of *C. elegans* is that the complete cell lineage (959 somatic cells in the adult hermaphrodite; 1031 in the adult male) of the species has been determined (Sulston and Horvitz, 1977). Hence, this serves as a powerful tool in helping researchers get a deeper understanding of the cellular cues underlying the specification of cell fate in an organism. Furthermore, it was observed that during the development of both sexes (Hermaphrodites and males), a large number of cells (131 in the case of hermaphrodites)

were found to undergo programmed cell death (Horvitz. 1999). Further research into this phenomenon revealed the existence of a genetic pathway regulating this process which was also found to play a conserved role in other organisms, including humans (Green and Evan, 2002), (Wickremasinghe and Hoffbrand, 1999). For the discovery of this programmed cell death pathway in *C. elegans* and for their pioneering work on genetics of organ development, researchers Sydney Brenner, Robert Horvitz and John Sulston were jointly awarded the 2002 Nobel Prize in Physiology or Medicine (Check, 2002).

Another useful feature of *C. elegans* is that it is possible to disrupt the function of specific genes with relative ease by means of a technique called RNA interference (RNAi) (Novina and Sharp, 2004).

Given the magnitude of knowledge on the development of *C. elegans*, research interests since the past decade have started to shift focus towards getting an understanding into the evolution of development by using *C. elegans* as a platform for performing comparative studies with other nematode species such as *C. briggsae*, *C. remanei*, *Pristionchus pacificus* etc. The nematode phylum is indeed rich in species, many of which similar to *C. elegans*, have been found to display invariant cell lineages and developmental processes (Sommer, 2005). Such a comparative study using diverse species would provide a comprehensive overview about the subtle developmental variations and changes in a phylogenetic context (Sommer, 2005), (Rudel and Sommer, 2003). In fact, studies conducted on certain key developmental processes of the nematode, such as the formation of the gonad, pharynx development, and male tail development have offered

valuable insights into the evolutionary changes that occur during nematode development (Sommer, 2005).

The development of the vulva (the organ used for egg laying) among various nematode species has also greatly captured the interest of researchers mainly because the signaling pathways involved in the formation of this organ have also been found to play prominent roles during the development of many other organisms including humans. An interesting aspect of vulval development which makes it a good model for comparative studies is the fact that despite the overall variability at the level of genome sequences, a vast majority of nematode species display an invariant pattern of vulval development (Sternberg, 2005).

1.3 Vulval development

The development of the hermaphrodite vulva in *C. elegans* is widely deemed to be a powerful paradigm to examine the interplay between signal transduction pathways during organogenesis. Vulval development occurs as a result of multiple cell-cell interactions and interactions between diverse cell signaling pathways such as the Ras, Notch and the Wnt pathways (Sternberg, 2005). In addition, it serves as a useful model to study tissue remodeling and commitment to fates among cell types (Sternberg, 2005).

The adult vulva is comprised of 7 distinct cell types (Vul A, Vul B, VulB1, Vul C, Vul D, Vul E and Vul F) (**Fig.1.1C**) which are derived from the divisions of three of six

equipotent precursor cells which assume either a primary or a secondary fate in response to an inductive signal arising from the anchor cell (located on the somatic gonad). Various developmental and genetic studies have revealed that components of signaling pathways such as the Ras, Notch and Wnt pathways play a pivotal role in the development of the vulva (Sternberg, 2005).

1.3.1 Stages in vulval development

Vulval development occurs through a series of five stages.

1. Generation of the vulval precursor cells (VPCs): During the L1 and L2 larval stages, six Pn.p cells along the ventral hypodermal region namely P3.p – P8.p are specified as the vulval equivalence group (Sternberg, 2005). The hox gene *lin-39* is a key determinant of the VPC group. *lin-39* is expressed in the Pn.p cells P3.p to P8.p. The loss of *lin-39* activity results in the lack of VPCs, because the presumptive VPCs fuse with the hyp7 epidermis (Salser et al., 1993), (Clark et al., 1993), (Eisenmann et al., 1998), (Gleason et al., 2002). Thus, *lin-39* has been recognized so far as having at least two prominent roles: preventing VPC fusion and stimulating cell division particularly among the P3.p – P8.p cells, since overexpression of *lin-39* does not confer competence to additional Pn.p cells such as P2.p or P9.p. Here Wnt signaling via BAR-1 β-catenin has been found to be essential to sustain expression of *lin-39* in the VPCs P (3-8).p (Sternberg, 2005), (Gleason et al., 2002), (Eisenmann, 2005).

2. Patterning of the VPCs: Once competence has been conferred to the VPCs, an inductive signal arising from the anchor cell along with lateral signaling among the VPCs induces specifically three VPCs namely P5.p, P6.p and P7.p to adopt vulval cell fates (**Fig.1.1B**) (Sternberg, 2005). The anchor cell is specified during the L2 larval stage from among two somatic gonadal cells. The vulval cell fates are primarily of two types, 1° and 2°, each of which produce distinct sets of progeny. The uninduced VPCs adopt a non vulval 3° fate and eventually fuse with the large syncytial epidermis hyp7 (Sternberg, 2005). In the wild-type hermaphrodite, VPCs adopt their fates in an accurate spatial pattern namely 3°-3°-2°-1°-2°-3° (**Fig.1.1A**) (Sternberg, 2005).

3. Specification of vulval cell fates: The adult vulva consists of 22 cells of seven distinct types, namely vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF, which diverge in their patterns of gene expression (**Fig.1.1C**) (Sternberg, 2005).

4. Anchor cell invasion: The anchor cell begins to undergo a slow invasion towards the descendents of P6.p during the mid L3 larval stage. During the late L3 stage, the basement membrane of the anchor cell breaches the gonadal and ventral epidermal basement membranes and makes contact with the two central P6.p granddaughter cells, namely the VulF cells. This invasion finally concludes 3.5 hrs into the L4 larval stage, where the anchor cell extends towards the center of the VulF cells and forms a hole in the epidermis (**Fig.1.2.C**) (Sternberg, 2005), (Sherwood and Sternberg, 2003), (Gupta et al., 2003).

5. Morphogenesis of the vulva: With the invasion of the anchor cell, the development of the vulva is almost complete (**Fig.1.2.**). In the final stage of vulval development, the seven different cell types of VPCs undergo invagination to give rise to seven distinct toroids, which connects to the uterus, and everts as the hermaphrodite molts to adulthood (Sternberg, 2005).

1.4 Cell signaling in *C. elegans* vulval development

The development of the vulva involves the coordinated involvement of at least 3 major signal transduction pathways, namely the Ras, Notch and the Wnt pathways. During the early L3 larval stage, an inductive signal arising from the anchor cell acts through epidermal growth factor receptor LET-23 [Ras pathway] to induce P6.p to adopt the 1° fate (**Fig.1.5A**). Next, lateral signaling via LIN-12 [Notch pathway] (**Fig.1.5B**) specifies 2° cell fates among the P5.p and P7.p cells (Horvitz and Sternberg, 1991). There also exists cross inhibition between these two pathways where the LIN-12 signaling turns on inhibitors of LET-23 signaling, and by the same token, the LET-23 signaling in turn up-regulates the ligand for LIN-12 and down-regulates the receptor LIN-12 (Sundaram, 2004), (Sternberg, 2005). In addition, a canonical Wnt signaling pathway has also been found to play a prominent role in regulating the vulval induction process of the VPCs (Eisenmann, 2005), (Gleason et al., 2002).

The canonical Wnt signaling pathway is one the few evolutionarily well conserved genetic pathways whose function is required extensively during an organism's

development, from the small soil nematode *C. elegans* to humans (Eisenmann, 2005). The Wnt family of secreted glycoprotein's is a group of highly conserved signaling molecules that have prominent roles in various aspects of development such as regulation of the cell-cell interactions during embryogenesis, mitogenic stimulation, cell fate specification, and differentiation (Cadigan, 1997).

The transcription factor β–catenin (Armadillo in flies) is one of the important effectors of the Wnt signaling pathway. In the absence of the Wnt ligand, the cytoplasmic β–catenin associates with scaffold proteins APC and Axin and serves as a substrate for phosphorylation by the kinases CKI and GSK3-β (Logan and Nusse, 2004). The phosphorylated β–catenin is then ubiquitinated and targeted for degradation by the proteosome (Logan and Nusse, 2004). On the other hand, when Wnt ligands bind to a Frizzled family receptor and a co receptor of the LRP-5/6/arrow family, the formation of the APC/Axin/CKI/GSK3-β complex is inhibited, thus leading to the stabilization of β–catenin and its translocation to the nucleus. Once in the nucleus, β–catenin interacts and associates with the TCF/LEF family of transcription factors leading to the transcription of several downstream gene targets. In the absence of a signal, the TCF/LEF factors interact with other transcription factors (e.g. Groucho, histone deacetylase) and bind DNA to repress transcription of downstream gene targets (**Fig.1.4.**) (Cadigan, 1997), (Logan and Nusse, 2004).

Mutations in genes involved in the Wnt pathway in humans have often been linked to several types of cancers such as T cell leukemia, Colon cancer, oral cell carcinoma etc (Logan and Nusse, 2004).

1.4.1 Non-canonical and canonical Wnt signaling in *C. elegans*

The non-canonical Wnt signaling pathway was the first well-characterized Wnt pathway in *C. elegans* (Han, 1997). This pathway was found to play a key role during early embryogenesis to ensure the proper development of the endoderm and mesodermal tissues by polarizing the EMS cell to divide into an anterior MS cell (which forms the tissues of the mesoderm) and a posterior E cell (which gives rise to the endoderm). Here, the key components of the canonical Wnt signaling pathway such as homologs of APC, GSK3β and TCF were found to behave in an opposite fashion compared to those studied in other species. This suggested that Wnt signaling function was basically different in *C. elegans* (Han, 1997).

Subsequent studies however, showed the existence of both canonical and non-canonical Wnt signaling pathways in *C. elegans* (Eisenmann, 2005). The central difference between the two pathways is that those processes that utilize the β–catenin homolog BAR-1 appear to use a canonical Wnt pathway similar to that found in other species, while those developmental processes that utilize the β–catenin homolog WRM-1 appear to use a non-canonical Wnt pathway that is fundamentally distinct from the non-canonical pathways found in vertebrates (Eisenmann. 2005). BAR-1, like other β–catenins, interacts directly

with the POP-1/TCF amino terminal domain, and can drive transcription in yeast (Korswagen et al., 2000), (Natarajan et al., 2001). On the other hand the WRM-1 β–catenin doesn't appear to directly interact with POP-1/TCF, and activate transcription (Korswagen et al., 2000).

Later during the developmental cycle, a canonical Wnt signaling pathway similar to that found in vertebrates and in *Drosophila* plays a pivotal role in the development of the hermaphrodite vulva and other processes such as the migration of the Q neuroblasts, specification of the fate of the P12.p cell fate etc. (Eisenmann, 2005), (Gleason et al., 2002).

1.4.2 Canonical Wnt pathway components in *C. elegans*

C. elegans possesses components of the canonical Wnt pathway very similar to those found in flies and vertebrates, with some important differences. First, similar to what has been found in other organisms, *C. elegans* possesses several genes encoding **Wnt ligands** (*lin-44, egl-20, mom-2, cwn-1 and cwn-2*), members of frizzled family of **Wnt receptors** (*lin-17, mom-5, mig-1 and cfz-2*) and **Disheveled** (*mig-5, dsh-1, dsh-2*). The *C. elegans* genome also contains homologs of the **Porcupine** (*mom-1*), **Casein Kinase I-α** (*kin-19*), **GSK-3β** (*gsk-3*), and **Axin** (*pry-1*). However, in contrast to other species, the *C. elegans* genome contains 4 genes encoding different **β-catenins** (*bar-1, wrm-1, hmp-2 and sys-1*). In addition, *C. elegans* appear to possess just a single **TCF/LEF** factor gene, namely *pop-1* and a single gene encoding the **APC**, namely the *apr-1* (Eisenmann, 2005).

1.4.3 Role of the canonical Wnt pathway in vulval development

As mentioned earlier, the vulva is generated from three (P5.p, P6.p and P7.p) of six equipotent vulval precursor cells, P3.p – P8.p. The canonical Wnt signaling pathway allows VPCs to adopt one of the three above mentioned cell fates. When this pathway is inactivated (seen in case of *bar-1, egl-20, cwn-1, lin-44* loss of function), many of the VPCs adopt the non-vulval Fused fate (F) (Eisenmann, 2005). On the other hand, when constitutively activated (as seen in *pry-1* loss of function), the Wnt pathway ectopically induces extra VPCs (which under normal conditions would adopt the 3° fate) to adopt vulval fates (Eisenmann, 2005). The hox gene *lin-39* is one of the key targets of this pathway. In *bar-1*/β-catenin mutants, *lin-39* expression is compromised causing many VPC's to adopt the F fate (Eisenmann, 2005). Overactivation of the Wnt pathway on the other hand, causes ectopic expression of *lin-39* resulting in extra VPCs adopting induced fates (Sternberg, 2005), (Eisenmann, 2005), (Gleason et al., 2002)

1.5 Phenotypes associated with vulval development mutants

Mutations in genes that influence vulval development frequently cause phenotypic defects which can be recognized under a stereo microscope. These phenotypes encompass a variety of morphological defects associated with vulval induction.

a) Morphological defects: Defects in vulval morphology include phenotypes such as the protruding Vulva (Pvl) (Seydoux et al., 1993), (Eisenmann and Kim, 2000) (**Fig.1.3C**),

the EGg-Laying-defective (Egl), marked by the inability of hermaphrodites to lay eggs (**Fig.1.3D**) (Trent et al., 1983).

b) Vulval induction defects: The phenotypes associated with defective vulval induction fall into two classes. First, mutations that diminish the activity of the Ras, Wnt pathway cause what is known as a VULvaless (**Vul**) phenotype (**Fig.1.3D**).Vulvaless hermaphrodites lack a functional vulva and as a result the fertile eggs that they have hatch internally, and in due course of time the progeny devour and escape from the corpse of their mother. On the other hand those mutations that constitutively activate the Ras, Wnt pathway result in a MUltiVulva (**Muv**) phenotype (**Fig.1.3B**). These multivulva hermaphrodites have in addition to the normal vulva other extra ventral protrusions, each a pseudovulva formed from the induction of VPCs which normally should not take up a vulval fate (Eisenmann, 2005), (Horvitz and Sternberg, 1991).

The present study introduces yet another vulval phenotype in *C. briggsae* that has not been previously described. This phenotype will be referred to as the **Muv-Vul** phenotype where mutant animals possess characteristic features of both Multivulva and Vulvaless mutants (**Fig.1.3E**).

1.6 *C. briggsae*, a comparative model to *C. elegans*

A central issue today in developmental research of nematodes, is in understanding how different signaling inputs are integrated in the specification of VPC fates during vulval

development. The nematode *C. briggsae* is a natural companion to *C. elegans* because of its morphological similarities coupled with its hermaphroditic life style and ease of maintenance in laboratory conditions (Gupta et al., 2007). Evolutionary studies reveal that the two species have diverged from a common ancestor around 100 million years ago (Gupta et al., 2007). The fact that these two nematode species share seemingly identical morphologies inspite of a great deal of variation in their genomes sequences allows for the exploration into the functioning of conserved genetic pathways during development. A recent study aimed at understanding the evolutionary variations in the *Caenorhabditis* genus suggests that although the molecular network of Ras and Notch signaling are conserved between *C. elegans* and *C. briggsae* during vulval development, there exists a cryptic (silent) evolution among the these intercellular networks governing vulval development within the *Caenorhabditis* genus (Felix, 2007). The availability of genome sequences for both nematode species and the amenability of *C. briggsae* to genetic manipulations make it highly suitable for us to carry out comparative genomic studies between the two nematode species (Gupta et al., 2007). Given that morphologically, *C. briggsae* is almost identical to *C. elegans,* while their genome sequences differ greatly raises the obvious question as to why these changes in the genome are not reflected in significant morphological or behavioral differences. It is indeed possible that perhaps such features are under a strong evolutionary selective pressure that has brought forth certain compensatory changes in the genome (Gupta et al., 2007). Alternatively, it is also possible that these two nematode species indeed show these differences in certain other developmental and physiological processes, which have yet been studied in detail.

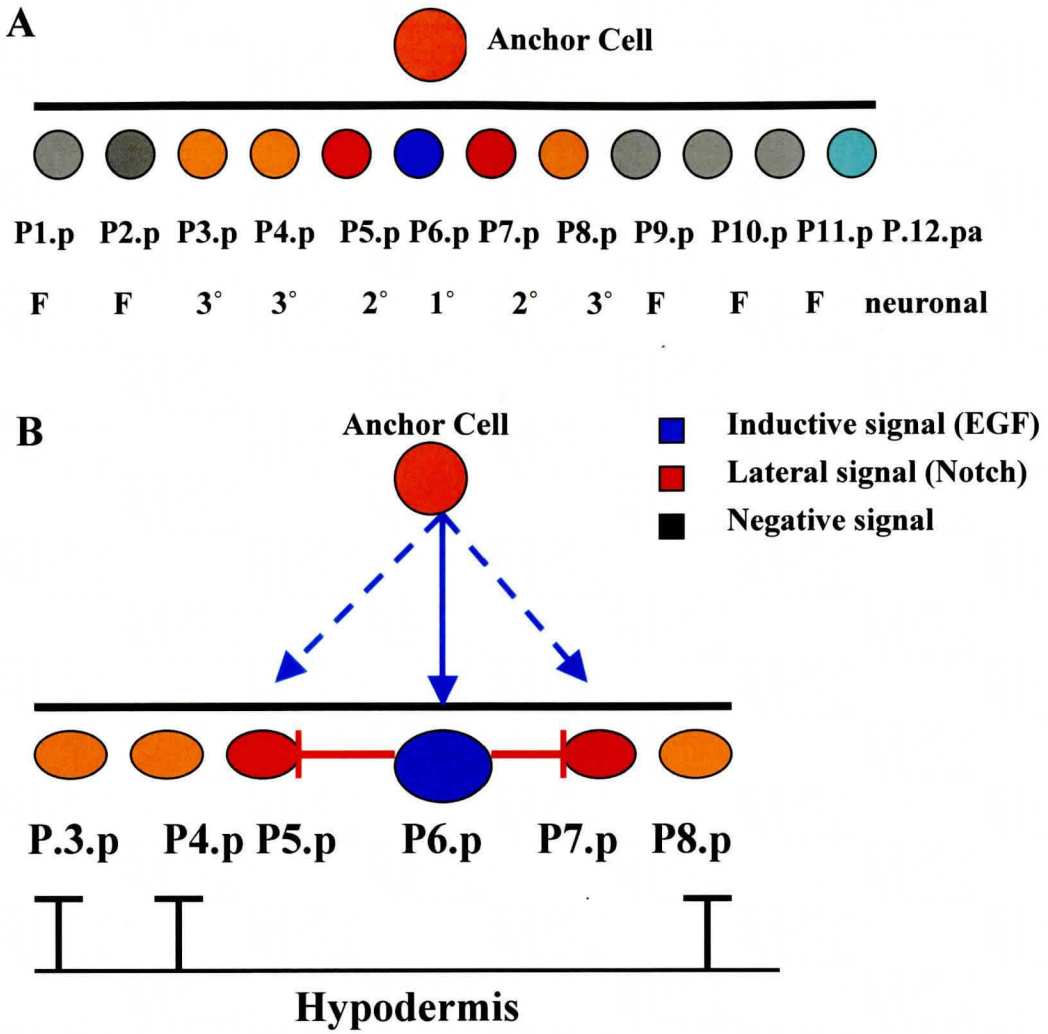
The present study aims at understanding the genetic regulation governing the development of the hermaphrodite vulva by the Wnt signal transduction pathway in *C. briggsae.* I have studied the role of the canonical Wnt signaling pathway in *C. briggsae* vulval development. The findings from this study suggest that the functions of some of the key regulators of the Wnt pathway have diverged from *C. elegans*. Future work would further our understanding on the genetic mechanisms governing the formation of a morphologically identical tissue and also offer insights into some of the subtle genetic variations that may exist in the regulation of the Wnt pathway between *C. elegans* and *C. briggsae.*

Figure.1.1: Vulval cell fate specification

(A) Shows the vulval cell-fate specification in *C. elegans*. The cells P (1, 2, 9–11) .p (gery colored cells) do not assume any vulval cell fate and eventually fuse with the underlying hypodermal syncytium hyp7. The cells P(3–8).p on the other hand form a vulval equivalence group and adopt one of three induced cell fates namely 1°, 2 ° and 3 °. The P (3, 4, and 8).p cells remain epidermal and adopt a 3 ° fate (**yellow ovals**). The P (5, 6 and7).p cells adopt a 2 ° fate and give rise to the anterior and posterior part of the vulva (**red ovals**). The P6.p cell adopts a 1° fate and forms the central part of the vulva (**blue oval**). The anchor cell (**AC, orange circle**) is seen just above the P6.p, cell, located on the somatic gonad. This cell later induces the vulva formation and makes contact with the progeny of cell P6.p.

(B) Provides a schematic overview of the various signaling interactions that occur during the formation of the vulva in *C. elegans*. The process of vulval induction starts with an inductive signal mediated by the EGF pathway that originates from the anchor cell (**blue arrows**). The P6.p cell upon receiving the inductive signal, signals its neighbors the P5.p and P7.p cells to adopt a secondary cell fate by means of 'lateral signaling' mediated by the LIN-12/Notch pathway (**shown by red arrows**). A third, negative signaling mediated by the Nurd complex (arising from the underlying hypodermal hyp7 cell) (**black bars**) functions to prevent any inappropriate vulva differentiation from taking place .

(C) Shows a schematic overview of the cell lineage pattern of the *C. elegans* P (5–7).p cells. The two VPCs P5.p and P7.p have identical lineages with opposite anterior posterior orientations such that they appear to be mirror images of each other. **L** represents the longitudinal division, **T** transversal and **N** nondividing cell.


A
Anchor Cell
P1.p P2.p P3.p P4.p P5.p P6.p P7.p P8.p P9.p P10.p P11.p P.12.pa
F F 3° 3° 2° 1° 2° 3° F F F neuronal
B
Anchor Cell
Inductive signal (EGF)
Lateral signal (Notch)
Negative signal
P.3.p P4.p P5.p P6.p P7.p P8.p
Hypodermis

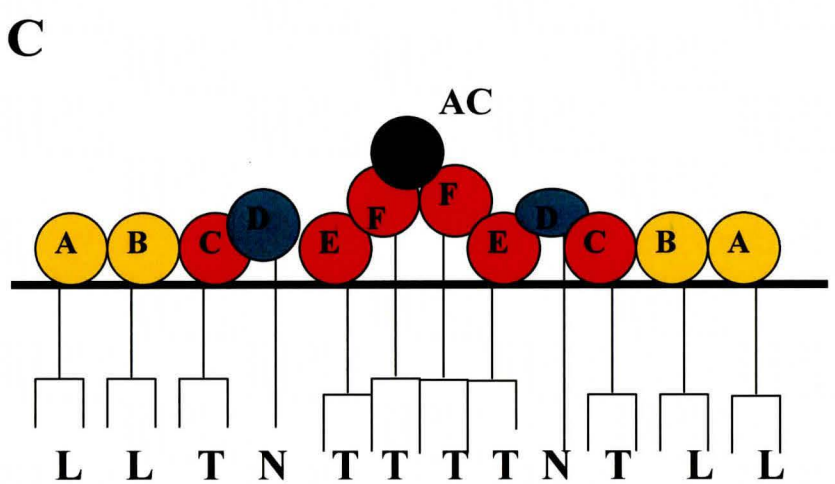
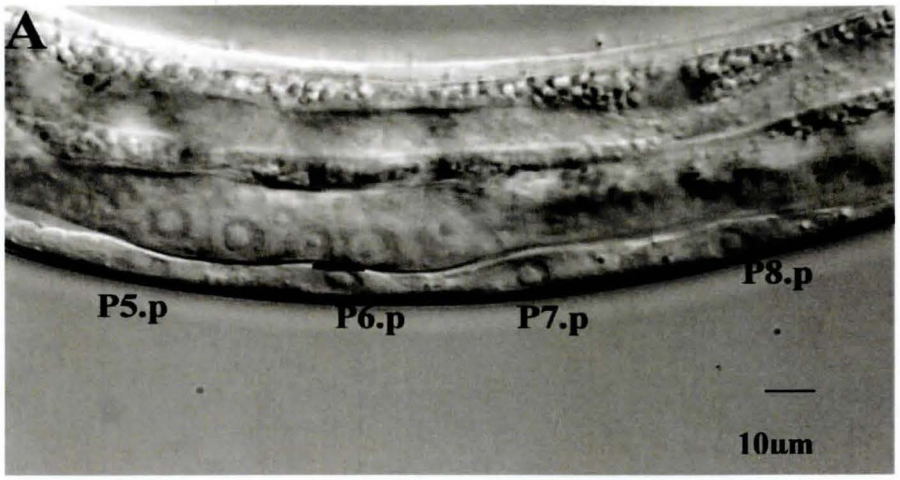
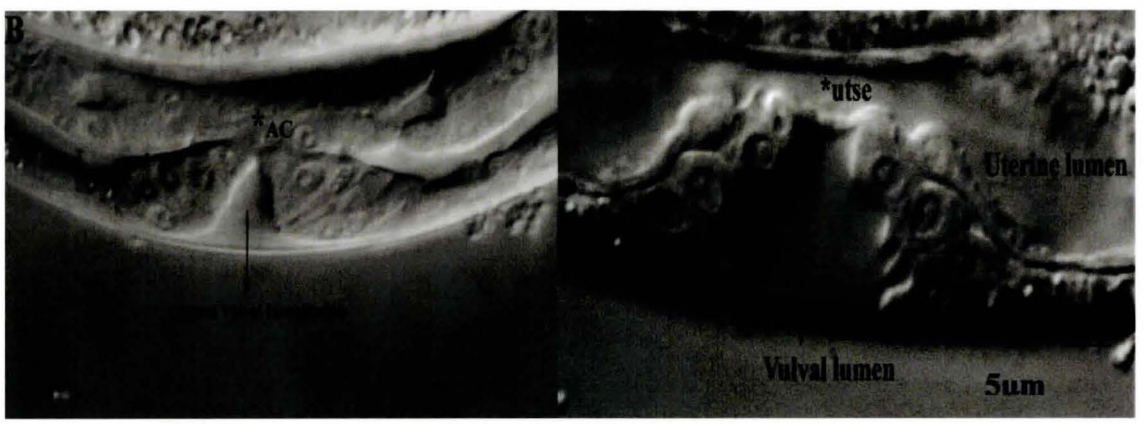

C
AC
A B C D E F F E D C B A
L L T N T T T T N T L L

Figure.1.2: Major landmarks during vulval morphogenesis, common to *C.elegans* and *C. briggsae*.

(**A**) Shows the position of the VPCs at the beginning of the L3 larval stage, just prior to the time of induction. (**B**) Represents the mid L3 to early L4 stage, where the central vulval lumen begins to form. (**C**) Represents the mid L4 stage, where the vulval lumen is fully formed and the anchor cell is no longer seen as it has fused with the uterine seam syncitium (utse). (**D**) Represents the adult stage, where the vulval development is complete with the formation of the slit and vulval lips.


A
P5.p
P6.p
P7.p
P8.p
10um


B
*AC
*utse
Uterine lumen
Vulval lumen
5um

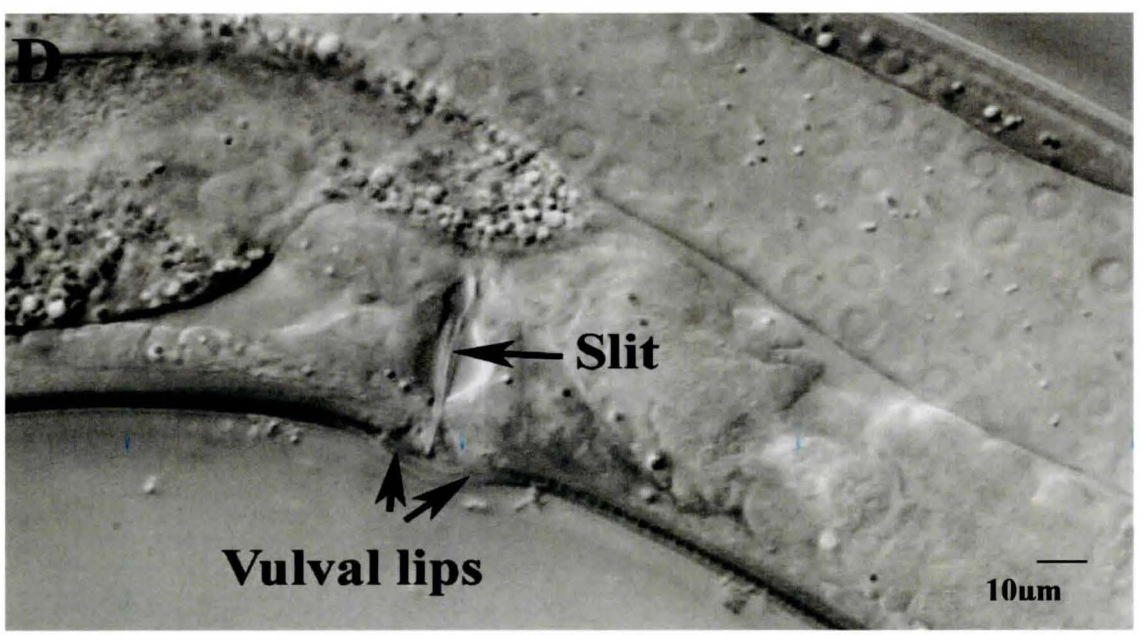

D
Slit
Vulval lips
10um

Figure.1.3: Vulval developmental mutants

(**A**) shows a typical wild type *C.elegans.* (**B**) A multivulva (Muv) mutant, where VPCs that normally do not assume any vulval fate are ectopically induced to adopt vulval fates, causing them to form pseudovulvae which appear as projections on the ventral body surface (represented by arrows). (**C**) The protruding vulva (Pvul) phenotype. (**D**) A Vulvaless (Vul) mutant showing a 'Bag of Worms' phenotype due to the inability to lay eggs caused due to the lack of a vulva. Incidentally this picture is also representative of another class of vulval mutants, namely the Egg laying defective (Egl) mutants. (**E**) A novel vulval phenotype seen in *C. briggsae* where a simultaneous co expression of Muv-Vul phenotypes is seen in the same organism. This phenotype is characterized by VPCs anterior to P6.p adopting induced cell fates while those VPCs posterior to P6.p adopt a non vulval fate.

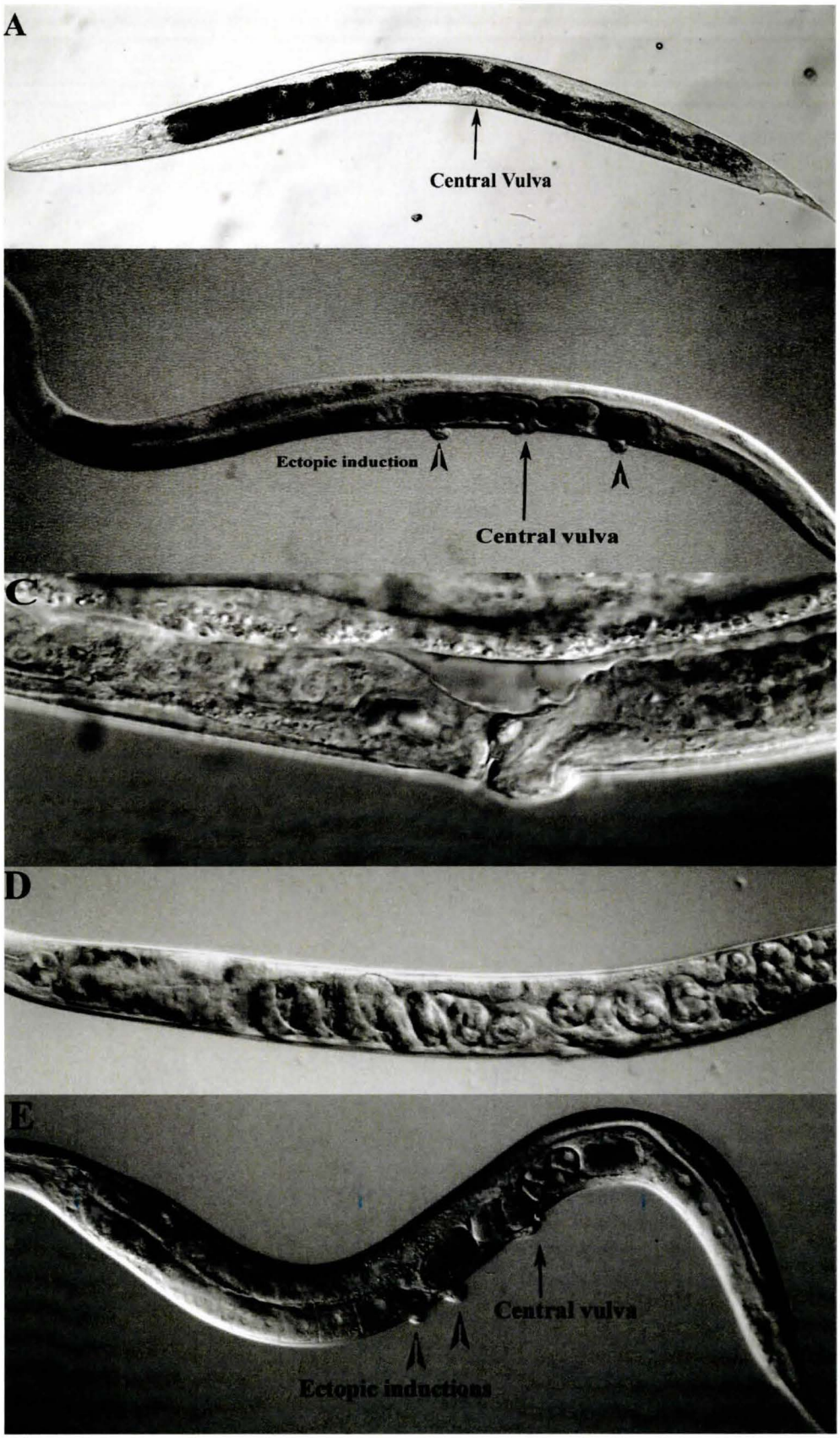
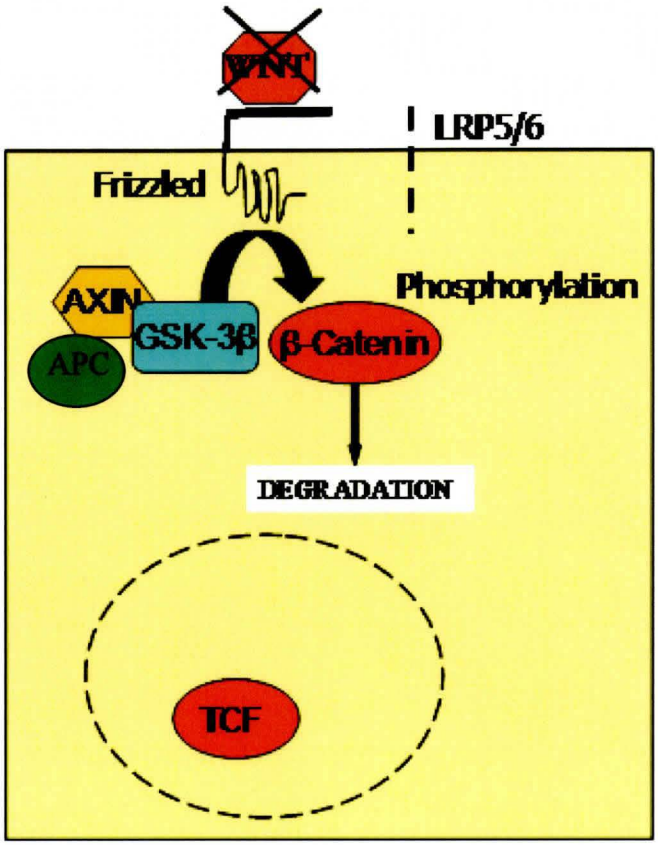

A
Central Vulva
Ectopic induction
Central vulva
C
D
E
Central vulva
Ectopic inductions

Figure.1.4: The canonical Wnt signal transduction pathway- An Overview

(**A**) in the absence of the binding of a Wnt ligand to a frizzled receptor, the cytoplasmic β–catenin associates with scaffold proteins APC and Axin and serves as a substrate for phosphorylation by GSK3-β. The phosphorylated β–catenin is ubiquitinated and targeted for degradation by the proteosome. (**B**) On the other hand, When Wnt ligands binds to a Frizzled family receptor and a co receptor of the LRP-5/6/arrow family, the formation of the APC/Axin/GSK3β–complex is inhibited, thus leading to the stabilization of β–catenin and its translocation to the nucleus. Once in the nucleus, the β–catenin then interacts and associates with TCF/LEF family transcription factors which then lead to the transcription of several down stream gene targets.

A


WNT
LRP5/6
Frizzled
AXIN
APC
GSK-3β
β-Catenin
Phosphorylation
DEGRADATION
TCF

B

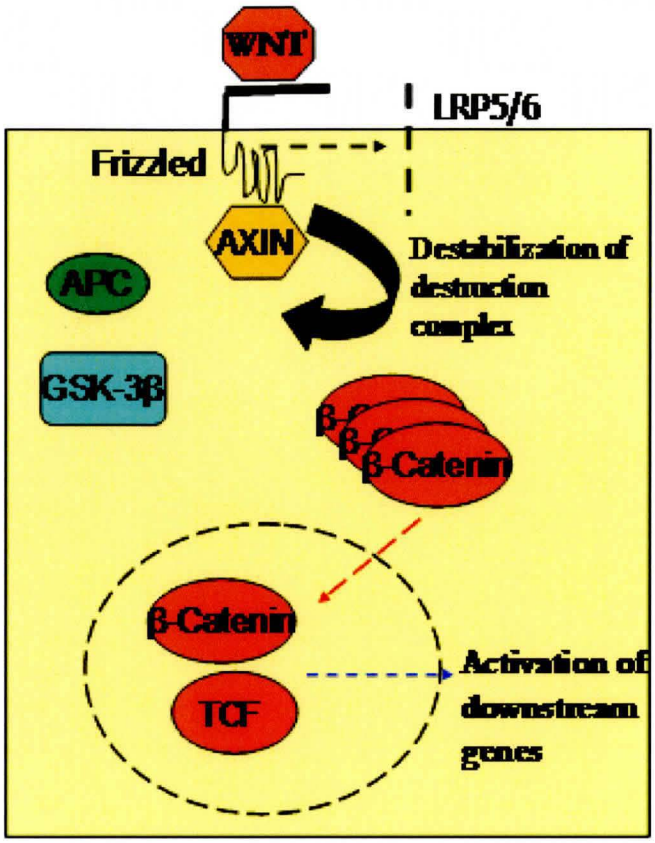
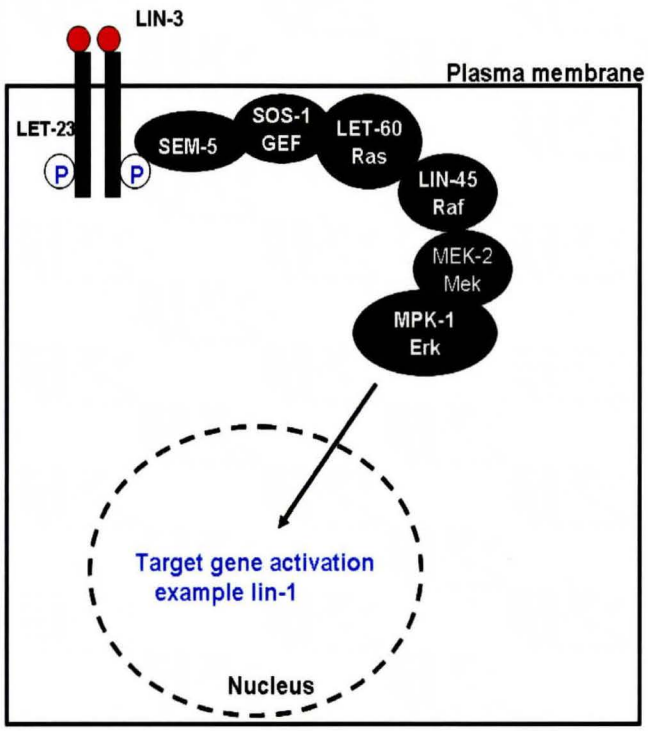

WNT
LRP5/6
Frizzled
AXIN
Destabilization of
destruction
complex
APC
GSK-3β
β-Catenin
β-Catenin
TCF
Activation of
downstream
genes

Figure.1.5: The Ras and LIN-12/Notch signaling pathways

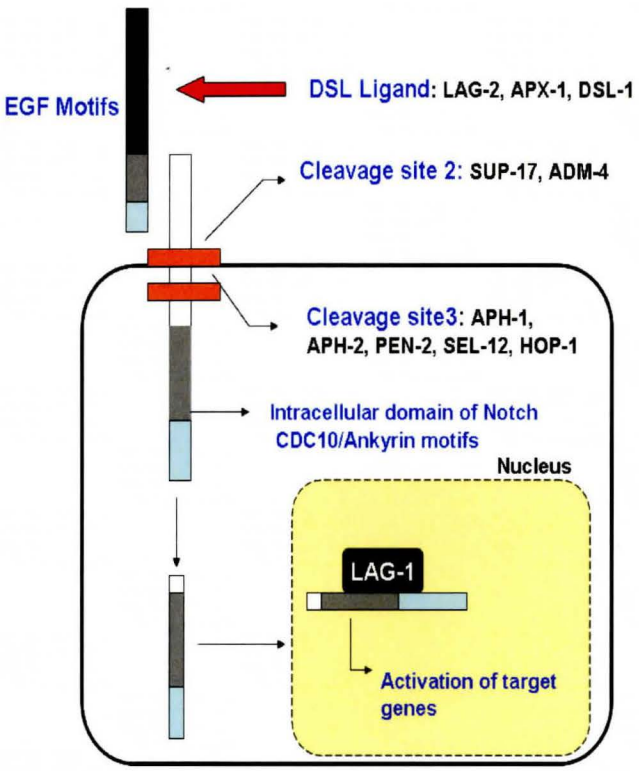
(A) Provides and overview of the Ras pathway. Upon the binding of the EGF ligand LIN-3, the RTK receptor LET-23 dimerizes and autophosphorylates it's C-terminus. The phosphorylated tyrosine residues now serve as docking sites for adaptor proteins such as the SEM-5 (Grb-2 in mammals). The adaptors in turn recruit a Guanine nucleotide exchange factor, SOS-1 in order to activate the LET-60 Ras. Upon activation, LET-60 binds to LIN-45 Raf and activates its kinase activity. LIN-45 next phosphorylates and activates MPK-2 which then activates the MAP kinase MPK-1. The activated MPK-1 can now move into the nucleus and promote the activation of several target genes (Sundaram, 2006).

(B) Provides an overview of the LIN-12/Notch pathway. The ligands for the LIN-12/Notch include APX-1, LAG-2 and DSL-1, members of the DSL family of ligands. These ligands contain either one or more EGF motifs, and in addition also contain a unique amino terminal motif. The binding of the ligand to the LIN-12/Notch receptor causes the shedding of the Notch ectodomain, through cleavage at the "Cleavage site 2". Next, the remaining transmembrane protein is further trimmed via cleavage at the "Cleavage site 3". This brings about the release of the intracellular domain, which then translocates to the nucleus. Next, the intracellular domain physically interacts with LAG-1, a memebr of the "CSL" family(derived from Mammalian CBF1 and *Drosophila* Su(H) and the LAG-1 from *C. elegans*). This interaction is facilitated by the CDC10/Ankyrin motifs on the intracellular domain of Notch. This interatcion between LIN-12/Notch and LAG-1 brings about the activation of downstream gene targets (Greenwald, 2005)

A


LIN-3
Plasma membrane
LET-23
P
P
SEM-5
SOS-1
GEF
LET-60
Ras
LIN-45
Raf
MEK-2
Mek
MPK-1
Erk
Target gene activation
example lin-1
Nucleus

B


EGF Motifs
DSL Ligand: LAG-2, APX-1, DSL-1
Cleavage site 2: SUP-17, ADM-4
Cleavage site3: APH-1,
APH-2, PEN-2, SEL-12, HOP-1
Intracellular domain of Notch
CDC10/Ankyrin motifs
Nucleus
LAG-1
Activation of target
genes

CHAPTER II: MATERIALS AND METHODS

2.1 Worm strains: The wild type reference strain used for *C. briggsae* was AF16. The wild type strain in *C. elegans* used for all the experiments was N2. The different mutant and integrant strains used in this study are as follows.

A) The following strains were obtained from CGC (*Caenorhabditis* genetics centre): N2, AF16, *Cel-lin-12(n952), Cel-pop-1(q645)* and *Cel-pry-1(mu38).*

B) The following strains were obtained from the Sternberg lab (Caltech): *Cbr-pry-1(sy5353).)* and *syIs53[unc-119(+) + pPGF11.07(lin-11::gfp)].*

C) The following strains were obtained from the Felix lab (Jacques Monod Institute): *mfIs5[Cbr-egl-17::gfp + Cel-myo-2::gfp], mfIs8[Cbr-zmp-1::gfp + Cel-myo-2::gfp], JU1078: mfEx[Cel-dlg-1::gfp+ Cel-myo-2::rfp], JU1018: mfIs42[Cel-sid-2 + myo-2::dsRED], mfIs29[Cel-lip-1::gfp + Cel-myp-2::gfp].*

C) The following strains were generated during the course of this study: *bhEx25[mec-7::gfp + Cel-myo-2::gfp], bhEx59[hs::Cbr-pry-1 + Cel myo-2::gfp], bhEx61[Cel-pry-1::gfp + Cel-myo-2::gfp], bhEx56[Cel-egl-5::gfp+ Cel-myo-2::gfp].*

2.2 Culture conditions: The general methods for culture and genetic manipulation of *C. briggsae* are as described (Brenner, 1974). Worm strains were allowed to grow on NGM agar plates which were seeded with OP50 (*E. coli* strains deficient for uracil). The strains used for all the experiments described, were maintained at 20°C.

2.3 Microscopy and cell ablations: Worms of larval stages (L1- L4) were mounted on 4% noble agar pads and observed using Nikon SMZ800, Zeiss Axioplan DIC (Nomarski) microscopes. To observe GFP expression, worms were viewed on a Zeiss Ax10 microscope fitted with a mercury lamp, using a standard fluorescein filter set and a Nikon DXM1200F digital camera. Cell ablations were performed using a laser microbeam by standard methods (Bargmann and Avery, 1995). Z1 and Z4 ablations were performed within 4 hours of hatching. The unablated, animals recovered from the same slides were used as a control. Ablated animals were identified by the absence of the germ line during the L3- L4 larval stage.

2.4 RNAi: The *C. briggsae JU1018* strain was used in all the experiments. *JU1018* worms are sensitive to RNAI by feeding, since they carry the *C. elegans sid-2* gene (*Cel-sid-2*), an intestinal luminal transmembrane protein that is required for environmental RNAi in *C. elegans* (Winston et al., 2007). Either L1 or L4 worms (depending upon the nature of the requirement of the gene of interest) were placed on agar plates containing 80ul of *E. coli* (HT115) transformed with the construct that would produce the double stranded RNA of the gene of interest. The phenotypic analysis was performed during the L4 larval stage of the worms.

2.5 Molecular biology

2.5.1 Constructs generated for the purpose of RNAi

1) *Cbr-mab-5* (pGLC12): This construct was generated by an undergraduate student Mark Hindle. A 0.6kb genomic fragment of *Cbr-mab-5* was PCR amplified from AF16 genomic DNA using the primers GL139/GL140. The amplified product was then cloned into the KpnI, SacI sites of the L4440 RNAi vector (Fire lab vectors) (**Fig.2.1**).

2) *Cbr-lin-39* (pGLC30): A genomic fragment (genomic position: 1- 2475) of 2.4 kb was amplified from the AF16 genomic DNA using the primer pair GL313/GL314. The amplified product was then cloned into the KpnI, SacI sites of the L4440 RNAi vector (Fire lab vectors). The construct was tested by a KpnI, SacI digestion to reveal the 2.4 kb insert and the 2.7 kb vector backbone (**Fig.2.2**)

3) *Cbr-sys-1* (pGLC31): A genomic fragment (genomic position: 1444-2340) of 0.9 kb was PCR amplified from the AF16 genomic DNA using the primer pair GL311/GL 312 (Down). The amplified product was then cloned into the KpnI, SacI sites of the L4440 RNAi vector (Fire lab vectors). The construct was tested by a KpnI, SacI digestion to reveal the 0.9 kb insert and the 2.7 kb vector backbone (**Fig.2.3**).

4) *Cbr-pop-1* (pGLC33): To construct the *Cbr-pop-1* RNAi construct, a 2.3 kb genomic fragment (genomic position: 11- 2273) was PCR amplified from the AF16 genomic DNA using the primer pair GL184/GL185. The amplified product was then cloned into the KpnI, SacI sites of the L4440 RNAi vector (Fire lab vectors). The construct was tested by a KpnI, SacI digestion to reveal the 2.3 kb insert and the 2.7 kb vector backbone (**Fig.2.4**).

2.5.2 Construct generated for rescue experiment

A) *Cbr-pry-1* heat shock rescue construct (pGLC29): This construct was generated by amplifying the full length 1.8kb *Cbr-pry-1* cDNA using primers GL301 and GL 302 and cloning it into the KpnI, SacI restriction sites of the pPD49.83 vector, which contains a heat shock promoter (Fire lab vectors). The construct was tested by a KpnI, SacI digestion to reveal the 1.8 kb insert, 3.8 kb vector backbone (**Fig.2.5**).

B) *Cbr-pry-1* heat shock experiment protocol: The *Cbr-pry-1* heat shock pGLC29 was injected at a concentration of 25ng/ul to generate stable transgenic lines in *C. briggsae*. The heat shock protocol was then administered as follows. Synchronized L1 stage larval worms of the *Cbr-pry-1(sy5353)* mutant carrying the rescue construct were placed onto Petri plates containing a healthy growing culture of *E. coli* [OP50] strain. The plates were then sealed with parafilm and then placed onto a water bath set at 31°C for duration of 24hrs. Following this, the worms were then scored when they were young adults for signs of rescue (exemplified by a lack of Muv phenotype).

2.6 Transgenics

All stable transgenic lines of nematodes used in this study were generated by means of microinjection as described by (Mello et. al., 1991).

2.7 Genetics

The following transgenic lines of *Cbr-pry-1(sy5353)* were generated using the cross scheme outlined in **Fig.2.6**.

*DY148: Cbr-pry-1(sy5353); JU*1078:*mfEx[Cel-dlg-1::gfp+ Cel-myo-2::rfp],*

DY163:*Cbr-pry-1(sy5353)BhEx59, Cbr-pry-1(sy5353); bhEx61.*

The transgenic strain *mfIs42 [Cel-sid-2 + myo-2::dsRED]; bhEx25* was constructed as outlined in **Fig.2.7**. This strain was then crossed into the transgenic *Cbr-pry-1(sy5353); mfIS42* background using the cross scheme outlined in **Fig.2.6**.

Figure.2.1: pGLC12

A

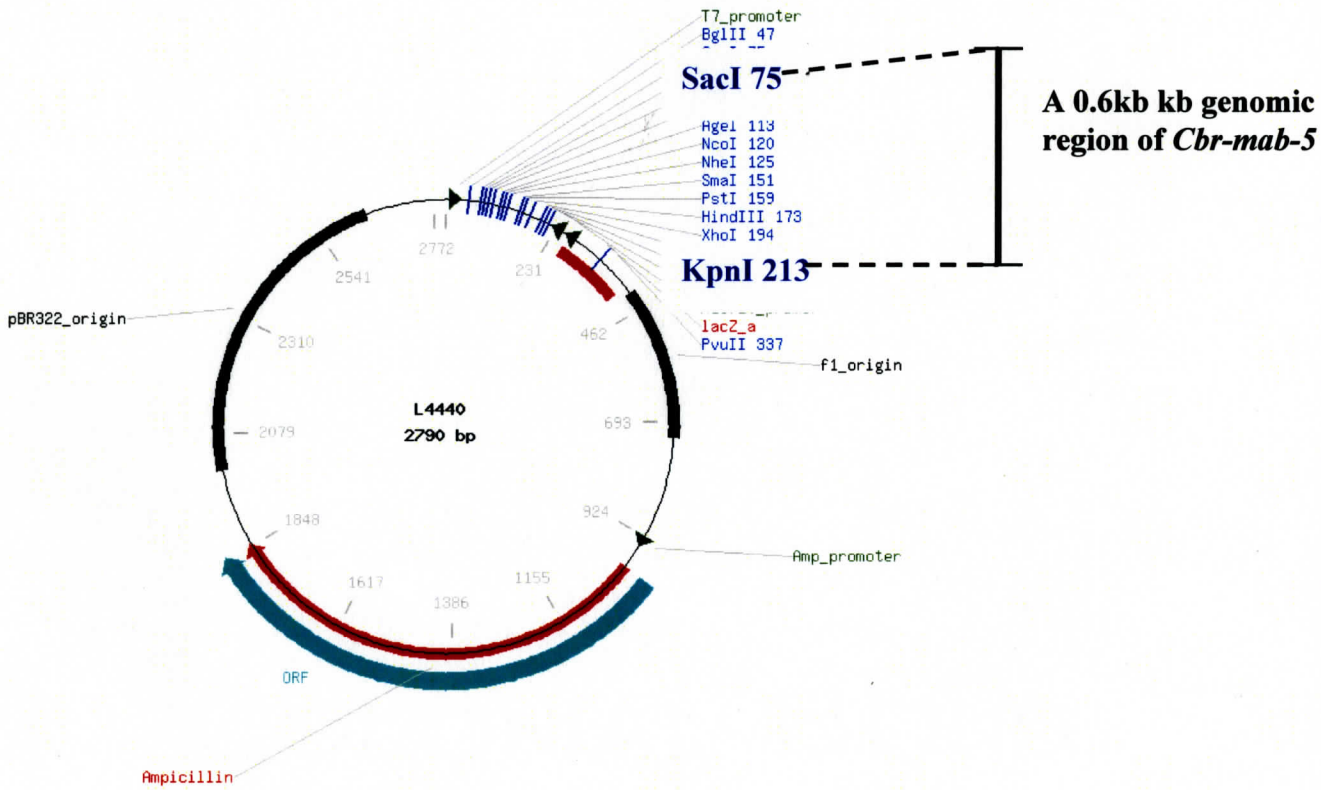
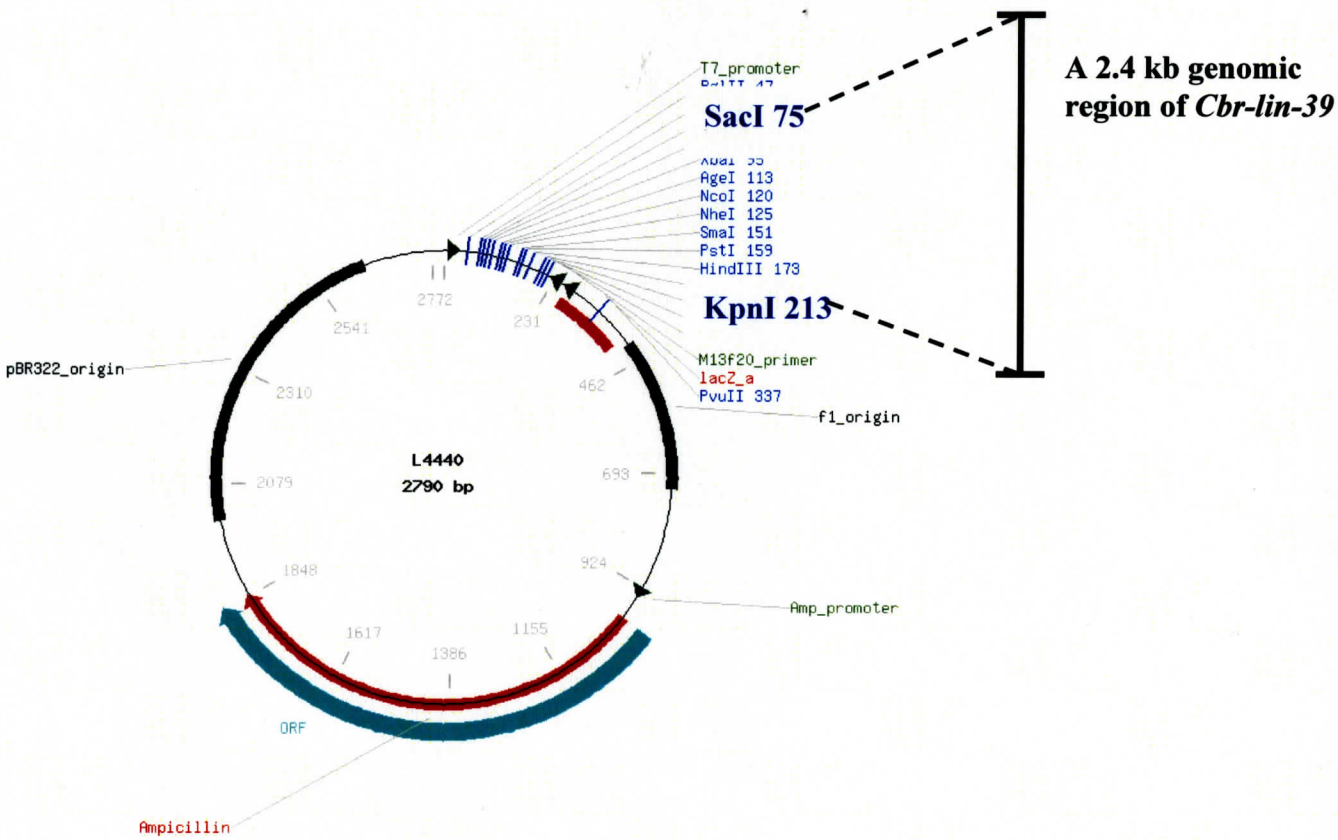


Figure.2.2: pGLC30

A

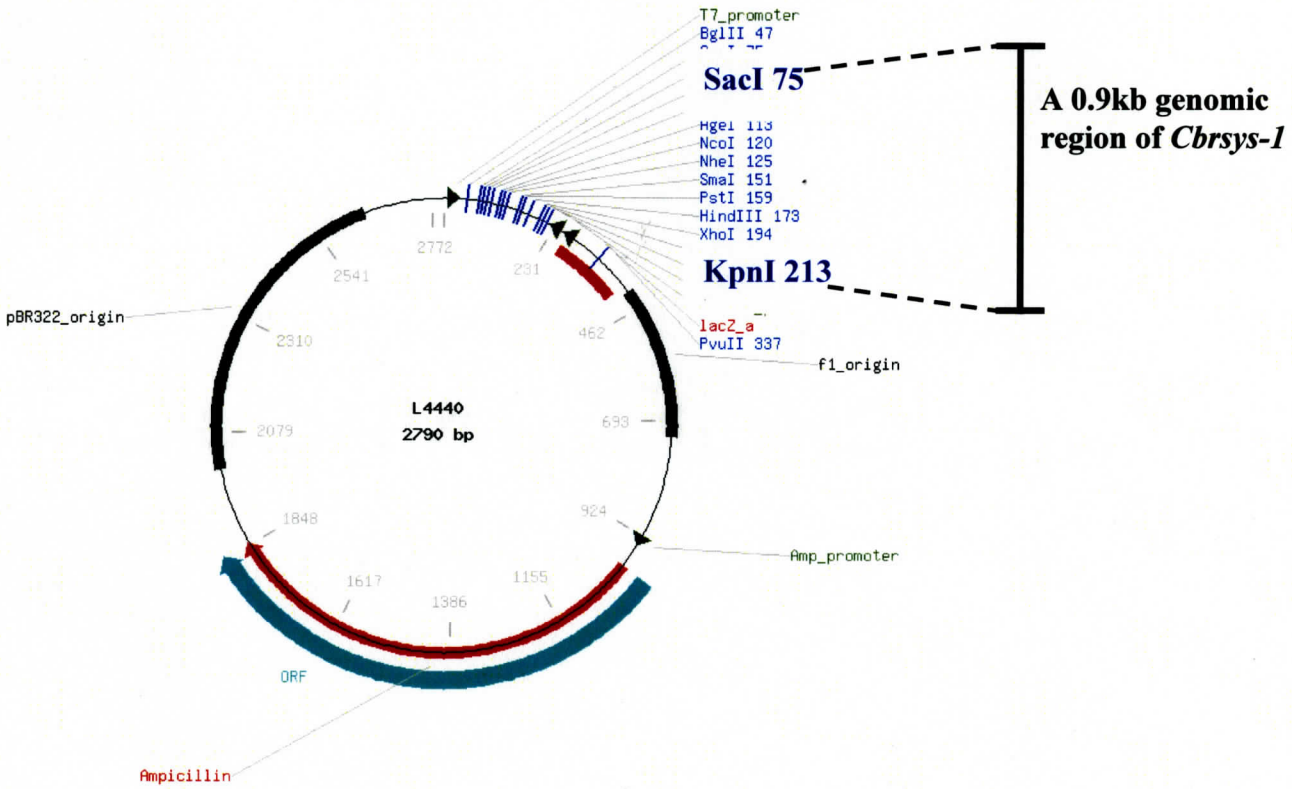


Vector image obtained from www.addgene.com

B

Figure.2.3: pGLC31

A.



Vector image obtained from www.addgene.com

B

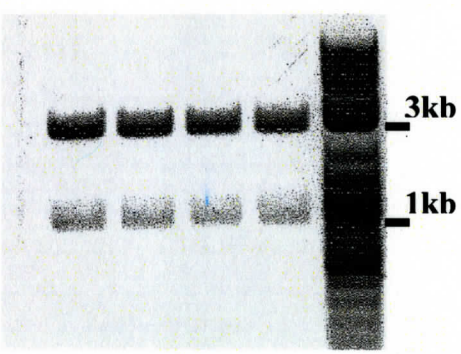
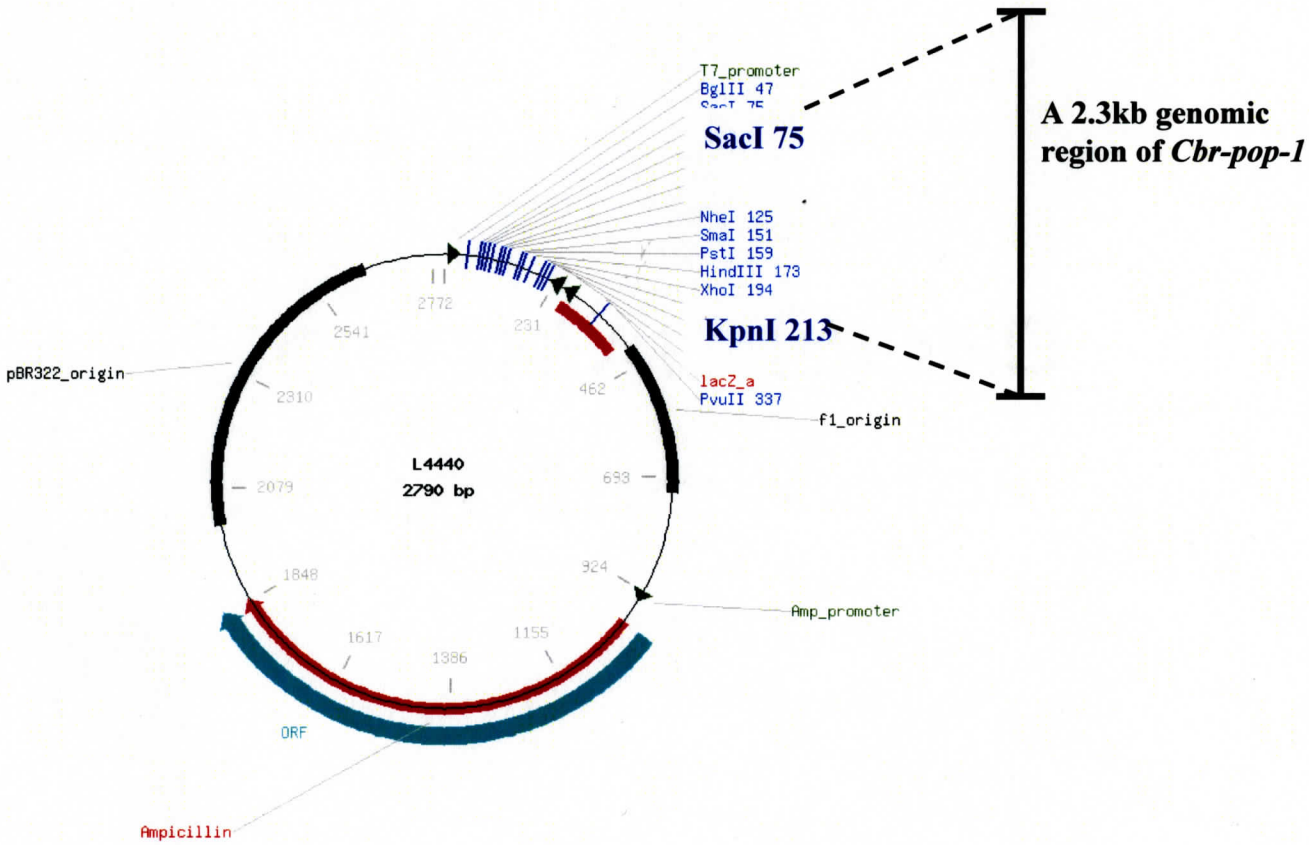


Figure.2.4: pGLC33

A.



Vector image obtained from www.addgene.com

B.

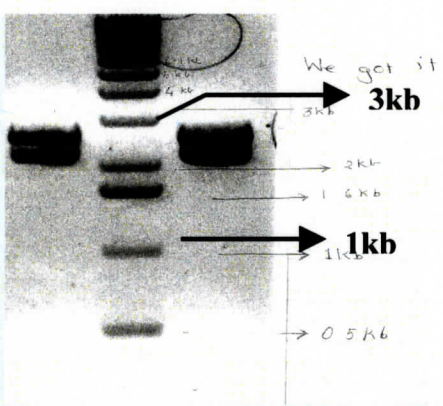
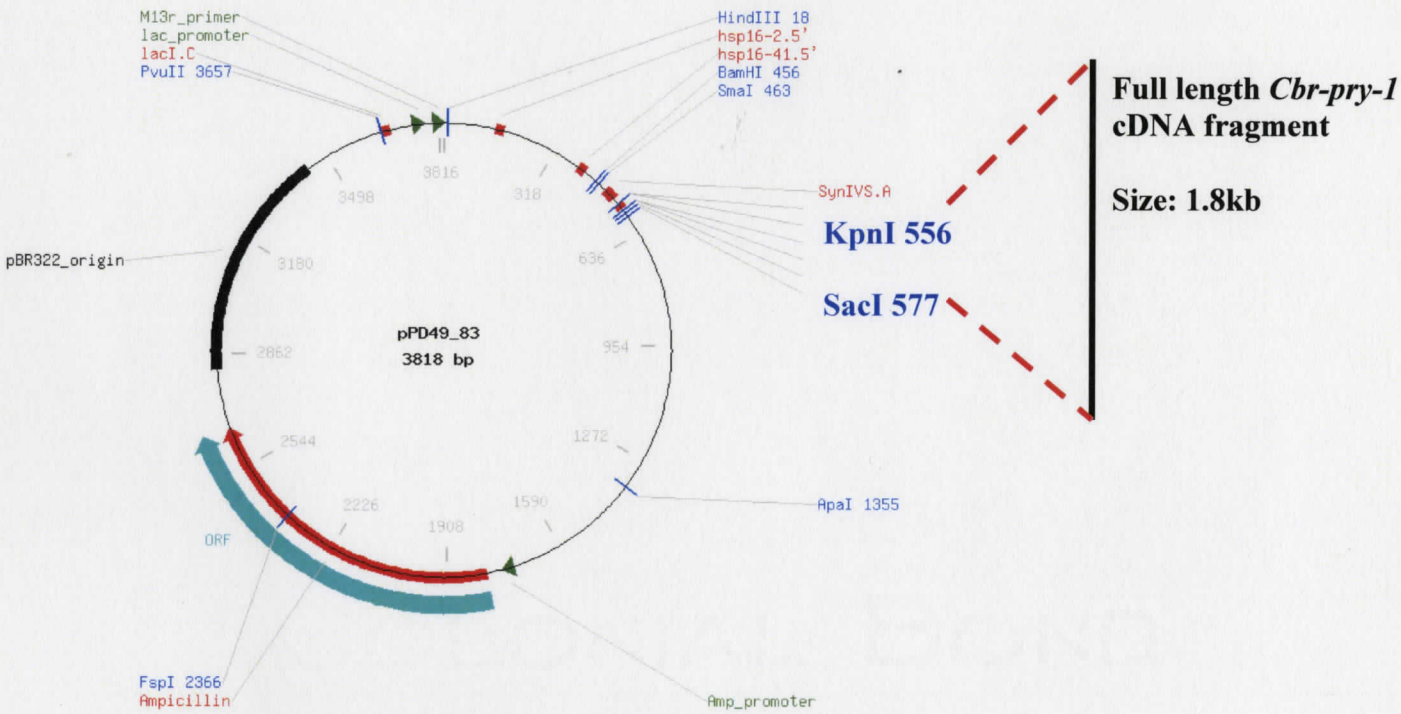


Figure.2.5: pGLC29

A



Vector image obtained from www.addgene.com

B

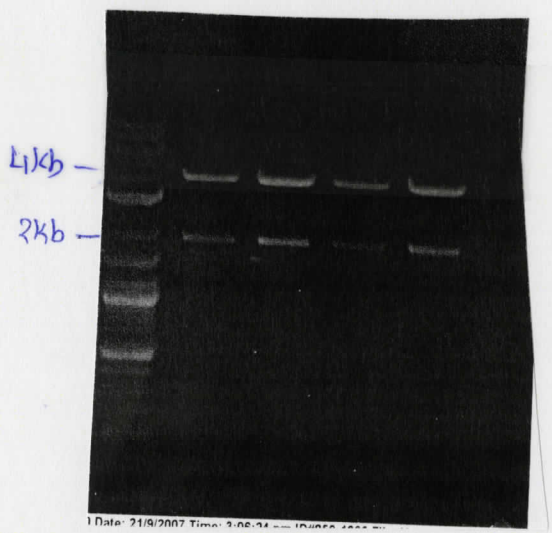


Figure.2.6: Genetic cross scheme I

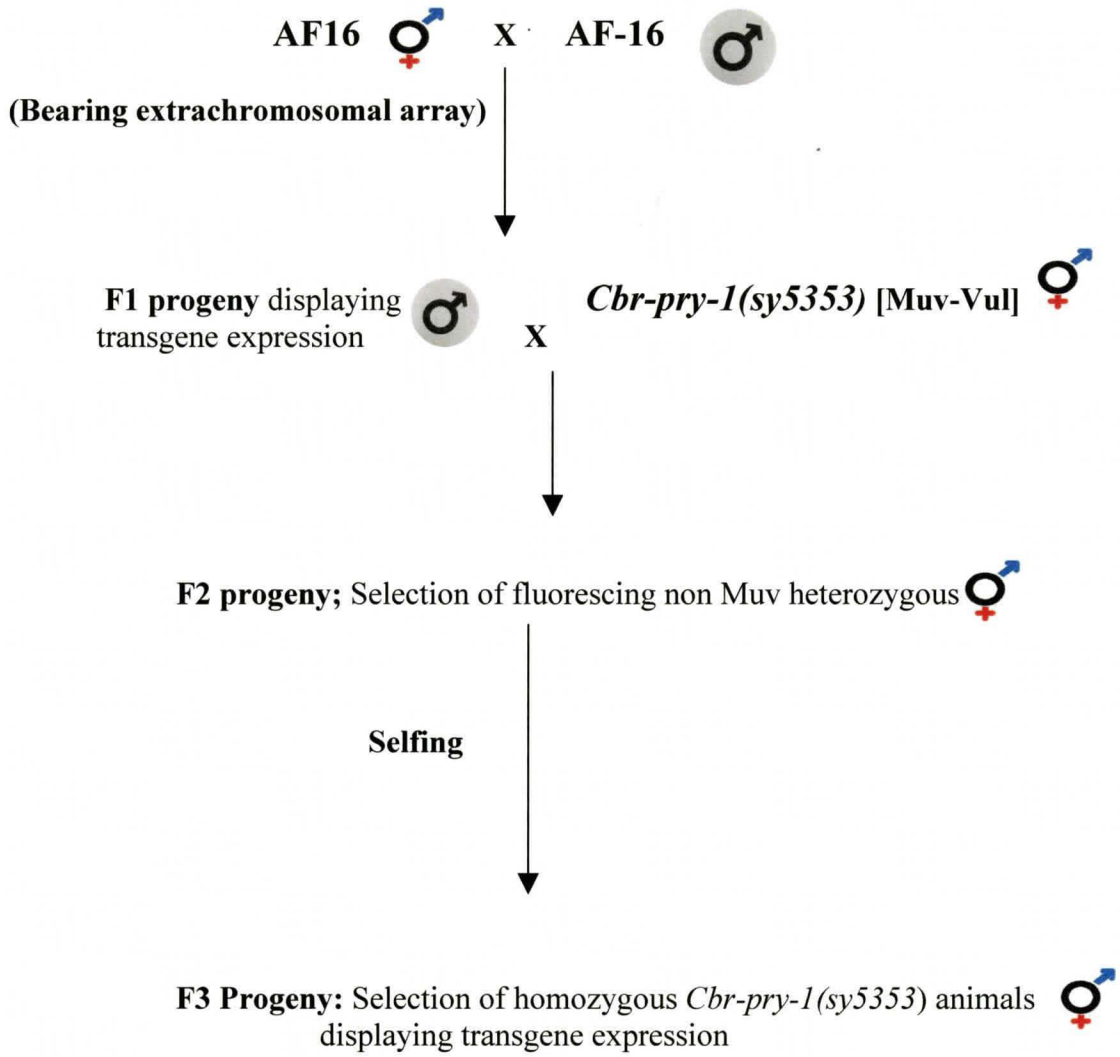
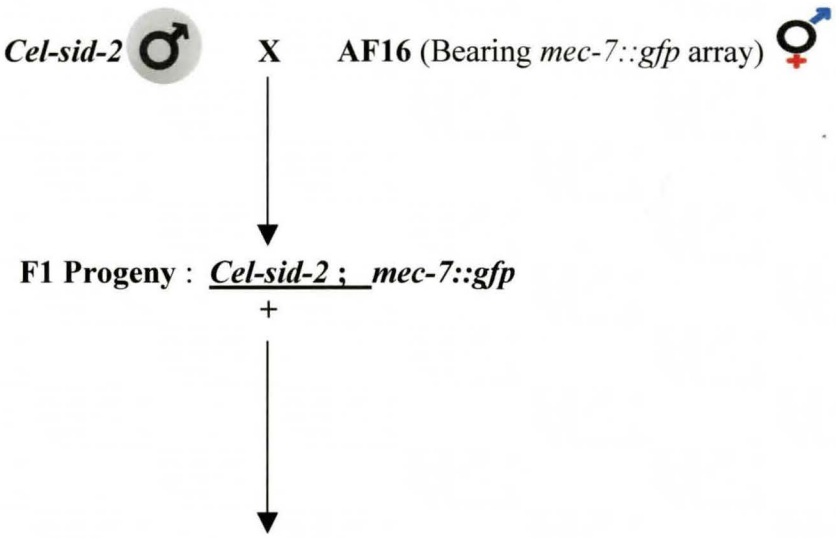


Figure.2.7: Genetic cross scheme II



F2 Progeny
Desired genotype: *Cel-sid-2; mec-7::gfp*

Next, worms with the genotype *Cel-sid-2; mec-7::gfp* were isolated based on their sensitivity to RNAi by feeding, because only those animals which are homozygous for *Cel-sid-2* are sensitive to RNAi by feeding.

Primers used in this study

Name	Gene	Sequence
GL301	*Cbr-pry-1*	CAGGGTACCATGGAGAGTGGACCATCATCTC
GL302	*Cbr-pry-1*	CAGGAGCTCATAGCGAATCTCGGCAGCAATTC
GL139	*Cbr-mab-5*	TTGGAGCTCATGAGCATGTATCCCGGATGGACAG
GL140	*Cbr-mab-5*	TTGGGTACCGAAGAATGCTGGTCATTTTGCTCGTC
GL141	*Cbr-pry-1*	TTGGAGCTCATGGAGAGTGGACCATCATCTCATCTC
GL142	*Cbr-pry-1*	TTGGGTACCGACCAATCAATCAGCATCTGTCAC
GL143	*Cbr-pry-1*	CTCATTTTGAAGCTCTCAATCTCAC
GL184	*Cbr-pop-1*	ACGAAGAGCTCGGCGATGAGGTGAAG
GL185	*Cbr-pop-1*	AAAGGTACCAACGCTCTTATCTCTCTTTCTCTTTGG
GL311	*Cbr-sys-1*	CTCGGTACCGGCATGAAACCCCAACAAATTCAA AC
GL312	*Cbr-sys-1*	CTCGAGCTCCAACTTACAAAAGGAAGAGG
GL313	*Cbr-lin-39*	CTAGGTACCATGACCACATCTACATCATCGC
GL314	*Cbr-lin-39*	CTGGAGCTCGCCTACAATGTTTTCTAAGCTCCC

CHAPTER III: Mutation in *Cbr-pry-1*(Axin) disrupts Wnt signaling in *C. briggsae*

3.1 Introduction

The discovery of Axin being the gene product of the mouse fused locus and an inhibitor of the Wnt signaling pathway was originally made in *Xenopus* embryos (Zeng et al., 1997), and was later shown to be capable of binding and forming a complex with GSK-3β and β–catenin (Ikeda et al., 1998). Since its initial discovery, a vast amount of *in vitro* and *in vivo* studies in other model systems, such as *Drosophila*, *C. elegans* and cultured mammalian cells have demonstrated that axin is indeed essential for the regulation of β–catenin (Clevers, 2006), (Fuerer et al., 2008). Axin plays the role of a scaffold protein in the multiprotein degradation complex (which includes other key components such as APC, GSK-3β and CKI-α) that facilitates the phosphorylation of β–catenin which then leads to its eventual degradation (Clevers, 2006; Fuerer et al., 2008).

Previous studies have found that mutations in axin give rise to a variety of health disorders in humans including cancer (as a result of constitutive activation of the Wnt pathway through the stabilization of β–catenin) (Logan and Nusse, 2004). A nonsense mutation in Axin2 for example, has been shown to cause severe tooth agenesis, a condition where several permanent teeth are found missing (Fuerer et al., 2008; Lammi et al., 2004). Apart from causing such tooth defects, mutations in Axin2 also predisposes the affected individuals to colon cancer (Clevers, 2006; Lammi et al., 2004). In addition,

axin mutations have been also been detected in cases of hepatocellular carcinomas (Logan and Nusse, 2004). Thus axin is viewed as a potential tumor suppressor based on its ability to downregulate Wnt signaling (Logan and Nusse, 2004). Hence these studies demonstrate that the deregulation of β–catenin degradation caused by axin mutations is an essential step in the onset of many types of cancers in humans. Taken together, these studies reveal that the Wnt signaling pathway plays an evolutionarily conserved role during development of both vertebrates and invertebrates.

Recent technological advances made towards genome sequencing have made it possible to get a deeper insight into evolution of conserved signaling pathways between different organisms. Fully sequenced genomes of some of the vertebrate and invertebrate organisms have offered interesting insights into the evolution of the Wnt signaling pathway. For example, we are now aware of the presence of 20 Wnt genes in humans and mice, 7 in *Drosophila* and 5 in *C. elegans* (Clevers, 2006; Logan and Nusse, 2004). Further studies have demonstrated that many of these orthologous genes have similar biological activities (Clevers, 2006; Logan and Nusse, 2004)

Interestingly, in certain primitive organisms, while components of the Wnt pathway exist, they are not however regulated by a Wnt signal. The amoeba *Dictyostelium* for example, has a gene called aardvark which is considered to be a vestige of the β-catenin (Grimson et al., 2000; Logan and Nusse, 2004). While the regulation of this gene is controlled by GSK-3β phosphorylation, there is no evidence for the presence of Wnt-like genes in this organism acting upstream of GSK-3β regulating the pathway (Logan and Nusse, 2004).

Hence it is conceivable that an ancient β–catenin based mechanism could have existed prior to the evolution of higher animals (Logan and Nusse, 2004). Moreover by the later addition of the Wnt and Frizzled genes, the β–catenin activity could have become subject to control from other cells, a key feature of organized multicellular life (Logan and Nusse, 2004). Thus, in light of these differences seen in the regulation of the Wnt signaling pathway, researchers have started to focus more on comparative studies between different model organisms to get an idea about full spectrum of the functional conservation of the Wnt signaling pathway.

As mentioned in chapter I, the canonical Wnt signaling pathway plays a pivotal role in the development of the hermaphrodite vulva in *C. elegans*. Mutations that disrupt Wnt signaling give rise to developmental abnormalities in the vulva. For example, a loss of function mutation in *Cel-pry-1*/Axin results in a Muv phenotype, as a result of constitutive activation of the Wnt pathway (Gleason et al., 2002). To get a better perspective of the evolutionary role of the Wnt signaling pathway in nematode vulval development, a genetic screen was performed by Dr. Gupta to isolate mutations affecting genes involved in the regulation of vulval development in the nematode *C. briggsae* (closely related to *C. elegans*). The screen *yielded* several mutants displaying excessive cell proliferation in the vulva. Initial phenotypic analysis and complementation studies conducted by B. Nagagireesh from the Gupta lab revealed that three mutants namely *sy5353, sy5411* and *sy5270* were in fact allelic. All three mutants (*sy5353, sy5411* and to a lesser extent *sy5270*) exhibited a unique pattern of vulval induction defect that is distinct from any of the other well known Muv mutants studied in *C. elegans,* namely the

Muv-Vul phenotype mentioned in chapter I. Other prominent developmental defects observed in these mutants include gonad arm folding defects, abnormalities associated with the male tail development such as the presence of several ectopic rays, defective ray morphology and the presence of pseudovulvae like structures along the ventral hypodermis (Nagagireesh Bojanala M.Sc Thesis). In addition to these, the mutant animals also display a transformation of the P11.p cell fate towards that of the P12.pa cell among the hermaphrodites.

Gene sequencing experiments conducted by Dr.Gupta revealed the identity of the gene underlying the phenotype of *sy5353* as *Cbr-pry-1/Axin.* A point mutation was uncovered between exons 5 and 6 that might disrupt their splicing. Hence *Cbr-pry-1(sy5353)* is most likely to be a loss of function mutation. The sequencing experiments also showed that the *Cbr-pry-1* locus encodes a protein of 610 amino acids that is 71% identical to its *C. elegans* counterpart. One of the main differences between the two orthologs is the absence of one exon in *Cel-pry-1* and the presence of two large introns in the C-terminal region of *Cbr-pry-1.* In addition, both proteins share highly conserved RGS and DIX domains. The genomic sequences of the *Cbr-pry-1* RGS and DIX domains display 83% and 84% identity with that of *Cel-pry-1.* Thus *Cbr-pry-1(sy5353)* serves as an excellent tool to launch a new line of scientific enquiry, in understanding the role of Wnt signaling in *C. briggsae* development. In addition, this also serves as a platform to perform comparative studies with *C. elegans.*

The purpose of this chapter is to provide a more detailed analysis of the *Cbr-pry-1(sy5353)* phenotype and highlight some of the key similarities and differences between the *Cbr-pry-1(sy5353)* and the *Cel-pry-1(mu38)* mutants.

3.2 Results

3.2.1 Phenotypic rescue of *Cbr-pry-1(sy5353)* mutant: Having identified a point mutation in the *Cbr-pry-1* gene, it was necessary to determine if it disrupts *Cbr-pry-1* function in *sy5353* animals. For this, a full length *Cbr-pry-1* cDNA was used to carry out the rescue experiment. The approach involved subcloning the *Cbr-pry-1* cDNA into the into the pPD49.83 vector which contains a heat shock promoter (hsp16-25', hsp16-415') and introduce this construct in the *Cbr-pry-1(sy5353)* mutants and examine the phenotype following heat shocks (Refer Chapter II-Materials and Methods for more details). The *Cbr-pry-1* heat shock was injected into wild type *C. briggsae* worms and stable transgenic lines carrying the *hs::pry-1* construct were obtained. These transgenic worms were subsequently crossed into the *sy5353* mutants.

The heat shock protocol was administered as described under the materials and methods chapter. The result showed that the overexpression of the *Cbr-pry-1* wild type gene was able to successfully rescue the mutant phenotype of *sy5353* suggesting that sy5353 allele disrupts the function of *Cbr-pry-1* **(Table.3.1)**.

3.2.2 Analysis of *pry-1* expression pattern: To get a better understanding of the functional conservation of *pry-1* between *C. elegans* and *C. briggsae*, we proceeded to compare the expression pattern of *pry-1* in *C. elegans* and *C. briggsae*. However, repeated attempts to amplify upstream sequences of the *Cbr-pry-1* gene proved unsuccessful and hence the *Cbr-pry-1* expression could not be analyzed. As an alternative approach, we decided to test whether the *C. elegans pry-1* gene could functionally replace its counterpart in *C. briggsae* and rescue the *Cbr-pry-1(sy5353)* mutant phenotype. To test this, transgenic lines of wild-type *C. briggsae* worms carrying a full length *Cel-pry-1* genomic DNA (Kindly provided by Dr. Korswagen) fused to a GFP reporter were generated. These transgenic worms were then crossed into the *Cbr-pry-1(sy5353)* mutant background and rescue was examined.

Analysis of *Cel-pry-1* expression pattern in *C. briggsae* showed that the gene is expressed from the time of embryogenesis (**Fig.3.1A, B**) as well as later larval stages (**Fig.3.1, 3.2**) and persists all the way into adulthood (**Fig.3.2**) . The expression of *Cel-pry-1::gfp* is observed in some of the P cells (progenitor cells which would eventually give rise to the neuronal Pn.a cells and hypodermal Pn.p cells) during the L1 larval stage (N = 10), thereby underscoring its possible requirement in the specification of the neurons and VPCs (**Fig.3.1C-F**). Analysis of the *Cel-pry-1::gfp* expression in the VPCs during later developmental stages shows that the expression is largely restricted to the primary lineage . Expression of *Cel-pry-1::gfp* is detected mainly in the P.6p cell during the L3 larval stage (**Fig.3.2B**). This expression continues to persist in the descendents of P6.p during the L4 and adult stages (**Fig.3.2C-F**). This seems to suggest that the *C. elegans*

pry-1 is capable of regulating vulval morphogenesis in *C. briggsae.* Nevertheless, the *Cel-pry-1::gfp* construct did not bring about a successful rescuing of the phenotype of the *Cbr-pry-1(sy5353)* mutant animals (**Fig.3.3**). Potential reasons underlying this apparent lack of rescue by the *Cel-pry-1::gfp* construct are discussed in greater detail under the discussion section.

3.2.3 A comparison of the *Cbr-pry-1(sy5353)* and *Cel-pry-1(mu38)* mutant phenotypes

3.2.3.1 Similarities

Analysis of the *Cbr-pry-1* phenotype has revealed some similarities and certain interesting differences from that of *Cel-pry-1*(*mu38*) mutants. Some of the similarities between these two mutants, such as the presence of a Muv phenotype, presence of ectopic rays in sy5353 males and a transformation of P11.p cell fate towards that of the P12.pa cell among the hermaphrodites have been mentioned earlier in this chapter. In addition, one other important similarity between the phenotypes of the *Cel-pry-1(mu38)* and the *Cbr-pry-1(sy5353*) mutants is a migration defect of the Q neuroblasts, discussed below. It is worth mentioning that this analysis had been performed earlier by B. Nagagireesh in the *Cbr-pry-1(sy5353*) animals. However, this experiment has been repeated for the present study in order to obtain data from more number of animals.

3.2.3.2 Analysis of Q neuroblast migration defect in *Cbr-pry-1(sy5353)* mutants: The Q cells, QL and QR are migratory neuroblasts born in identical A/P positions but on the opposite sides of the animal, namely QL on the left hand side and QR on the right (Korswagen et al., 2002). In wild-type animals, the QL, after a short posterior migration turns on *mab-5*, which in turn causes descendants of the QL cell (referred to as QL.d) to migrate towards the posterior (Korswagen et al., 2002; Maloof et al., 1999). On the other hand, *mab-5* remains turned off in the QR cell and its descendants (referred to as QR.d). Hence, the QR.d continue to migrate towards the anterior (Korswagen et al., 2002). Previous studies in *C. elegans* have shown that the regulation of *mab-5* in the migration of the Q neuroblasts to be directly under the control of the canonical Wnt signaling pathway (Korswagen, 2002). In *Cel-pry-1(mu38)* animals, both QL.d and QR.d migrate towards the posterior due to a gain of function of *mab-5* in the QR descendents (Korswagen, 2002).

As a next step, the migration pattern of the Q neuroblasts was examined in the *Cbr-pry-1(sy5353)* mutants. A previous study had shown the *mec-7::gfp* reporter to be a good marker of the QL.d and QR.d (Maloof et al., 1999). The Q neuroblast migration was studied by assaying the final Positions of the daughter cells of QL and QR namely AVM and PVM (Maloof et al., 1999). The results from this analysis support the previous findings by B. Nagagireesh. The *Cbr-pry-1(sy5353)* mutants display a migration defect of the Q neuroblasts similar to those observed in *Cel-pry-1* mutants (**Fig.3.4, Table.3.2**). Hence these finding suggests a conservation of the Wnt signaling pathway function between *C. elegans* and *C. briggsae* with respect to the migration of Q neuroblasts.

3.2.3.3 Differences

3.2.3.4 A novel Muv-Vul phenotype: As stated earlier, the *Cel-pry- 1* loss of function mutation results in a Muv phenotype where vulval precursor cells (VPCs) that normally do not adopt vulval cell fates are ectopically induced, as a result of overactivation of Wnt signaling. The *Cbr-pry-1* mutation on the other hand gives rise to homeotic transformations of VPCs resulting in a novel Muv-Vul (Vulvaless) phenotype. This is characterized by VPCs anterior to P6.p adopting induced fates while the VPCs posterior to P6.p adopt a non-induced fate. (**Fig.3.5, Table.3.3**). A hallmark feature of those cells that normally take up the non induced cell fate is that they eventually fuse with the underlying ventral hypodermal cell hyp7 (Kornfeld, 1997; Sternberg, 2005).

We wanted to determine whether the posterior VPCs P7.p and P8.p existed as fused or unfused cells. This was necessary because, if they indeed adopted a non-vulval fused fate, they should be concomitantly associated with the absence of cell junctions. The cell junction-associated marker *dlg-1::gfp* was used to detect the presence of any cell fusion taking place in the posterior VPCs (Bossinger et al., 2001). I found that in most of the cases, P7.p and P8.p did not fuse with hyp7 as revealed by the expression of *dlg-1::gfp* (**Fig.3.7, Table.3.4**). Thus unlike their anterior counterparts, the posterior VPCs in *sy5353* animals though unfused, still lacked the potential or competence to respond to the inductive signal. The apparent lack of competence among the posterior VPCs was further supported by the findings from laser ablation experiments. Previous work has shown that in the wild-type worms, isolated VPCs adopt primary and secondary cell fates in response

to the inductive signal (Sternberg, 2005). Similar experiments done in the *Cbr-pry-1* mutant animal's show that P7.p and P8.p cells tend to remain un-induced, suggesting they have lost their competence to respond to the inductive signal (B. Nagagireesh, M.Sc thesis).

3.2.3.5 Fate conversion of posterior Pn.p cells: An interesting observation made early on during the characterization of the *Cbr-pry-1(sy5353)* mutants was the morphological change of the entire posterior group of Pn.p cells (from P7.p- P11.p) towards that of the P12.pa cell, which normally adopts a neuronal cell fate. In wild-type animals, the Pn.p cells P7.p- P11.p are morphologically distinct from P12.pa. These cells are generally much bigger in size and display a more prominent nucleolus. However in the *Cbr-pry-1(sy5353)* mutants, the morphology of these posterior Pn.p cells bears a striking resemblance to that of the P12.pa cell (**Fig.3.6**).

The *lip-1::gfp* reporter in *C. elegans* is generally considered to be a good marker of the VPC fate. The expression of the marker is observed in the VPCs (but at a low level) until the L2 larval stage. During the early L3 stage, *lip-1::gfp* expression is up-regulated in the secondary VPCs P5.p and P7.p. For the duration of later stages that follow, the *lip-1::gfp* expression remains high in the descendants of P5.p and P7.p cells and somewhat low in the descendants of P6.p (Berset et al., 2001). Therefore we considered using *lip-1::gfp* as a marker to analyze the cell fate adopted by the VPCs in the *C. briggsae pry-1* mutant animals. The rationale for this approach was that if the posterior VPCs and other posterior Pn.p cells had in fact adopted a neuronal cell fate like that of P12.pa, then this

should be corroborated by a lack of *Cel-lip-1::gfp* expression in those cells. Accordingly transgenic lines of the *Cbr-pry-1(sy5353)* mutants carrying *Cel-lip-1::gfp* were generated and the expression in the VPCs was analyzed. However, the *Cel-lip-1::gfp* expression in the Pn.p cells of *C. briggsae* was not consistent. In addition, the *Cel-lip-1::gfp* expression was not restricted to the Pn.p cells either. The expression of *Cel-lip-1::gfp* was detected in both Pn.p cells as well as neuronal cells such as P12.pa (**Fig.3.8**). Hence although this observation offered an interesting insight into the regulation of *Cel-lip-1::gfp* in *C. briggsae*, it nonetheless did not serve our purpose for this line of enquiry and hence this analysis was not pursued further.

As an alternate approach, we decided to test another gene expression marker that would allow us to follow the P12.pa cell fate. The homeodomain transcription factor *Cel-egl-5* is expressed specifically in the posterior of the animal (Jiang and Sternberg, 1998; Kenyon, 1986). Moreover, it is considered to be a target of the Wnt signaling pathway, especially during the specification of the P12.p cell fate (Jiang and Sternberg, 1998; Kenyon, 1986). An *egl-5::gfp* reporter construct pLG7 described in a previous study highlights its use as a good marker primarily to follow the P12.pa cell fate (Teng et al. 2004). This suggested that analyzing the *Cel-egl-5* expression pattern in *Cbr-pry-1* mutants might provide an insight into the potential homeotic transformation of the posterior set of Pn.p cells. Stable transgenic lines of wild-type *C. briggsae* worms carrying an extrachromosomal array of the *Cel-egl-5::gfp* construct was generated. The transgenic worms were then crossed into the *sy5353* mutant background. However, during our preliminary analysis, I was unable to detect expression of the *Cel-egl-5::gfp*

reporter in neither the P12.pa cell nor in any other notable cell type in *C. briggsae*. This experiment has not been pursued further for the present moment.

Finally, it is also worth mentioning that in contrast to *Cel-pry-1(mu38)* mutants the *Cbr-pry-1(sy5353)* animals are generally healthier, and display WT body movements. In addition even though the *Cbr-pry-1* animals appear healthy, and display moments similar to WT, the Muv phenotype is almost fully penetrant (**Table.3.3**).

3.3 Discussion

The nematode *C. briggsae* is almost identical to *C. elegans* in morphology and shares an identical pattern of vulval development (Gupta et al., 2007). This feature coupled with our knowledge of the *C. briggsae* genome sequences and chromosomal assembly, has enabled us to carry out comparative studies to understand the evolution of conserved signal transduction pathways during vulval development. The identification of the *C. elegans pry-1/Axin* ortholog, a key component of the canonical Wnt signal transduction pathway, has allowed us to open up a similar line of enquiry in *C. briggsae* through which we can gain a deeper insight into how this pathway has evolved between closely related species.

Mutation in *C. briggsae pry-1*/Axin gives rise to a unique, previously undescribed phenotype where VPCs anterior to P6.p adopt induced fates while the VPCs posterior to P6.p adopt a non-induced fate. This simultaneous expression of both Multivulva (Muv) and Vulvaless (Vul) phenotypes in the same organism is referred to as the Muv-Vul

phenotype. This phenotype is suppressed by the introduction of the full length *C. briggsae pry-1 (Cbr-pry-1)* gene into the mutant animals thereby demonstrating the mutation in *Cbr-pry-1* alone to be the sole cause underlying the mutant phenotype. Furthermore, introduction of the *C. elegans pry-1 gene* into the *Cbr-pry-1(sy5353)* animals failed to rescue the *Cbr-pry-1* mutant phenotype. The *pry-1* gene in *C. elegans* is expressed in VPCs (p4.p – P8.p) at the time of vulval induction (Korswagen et al., 2002). Interestingly in *C. briggsae*, the *Cel-pry-::gfp* expression was observed in only one of the VPCs namely the P6.p at the time of vulval induction. The expression of GFP continues to persists in a subset of the descendents of P6.p namely VulF all the way up to the adult stage. It is unclear at this point as to why the *Cel-pry-1::gfp* rescue failed to occur in the *Cbr-pry-1(sy5353)* mutants.

A few lines of arguments can be brought forth to explain the apparent lack of rescue by the *Cel-pry-1* gene in the *Cbr-pry-1(sy5353)* mutants. First, GFP expression could not be detected in any of the VPCs other than P6.p during the time of vulval induction and during later stages. This loss of *pry-1* expression in the other VPCs may be a reason why the ectopic vulvae continue to persist in these mutants. Second, it may also be the case that the amount of protein produced by the *Cel-pry-1gfp* construct is inadequate to bring about a successful rescue. An experiment that can resolve this issue would be to test the efficiency of the *Cel-py-1::gfp* construct in rescuing the *Cel-pry-1(mu38)* phenotype. Moreover, given the amount of conservation between the *Cel-pry-1* and *Cbr-pry-1* locus (71% identity), the fact that the *Cel-pry-1* is unable to compensate for *Cbr-pry-1* suggests a possible alteration in regulatory elements governing the function of these two genes.

The phenotypic analysis of the *Cbr-pry-1(sy5353)* mutants highlights many similarities with that of the *Cel-pry-1* mutants and also certain intriguing differences. The nature of these differences between the two mutant species emphasizes a potential disparity in the regulation of *pry-1/Axin* between *C. elegans* and *C. briggsae*. The presence of an anterior Muv phenotype and a posterior Vul phenotype in the *sy5353* mutants seems to suggest that the Wnt signaling pathway has evolved in *C. briggsae* to somehow play a dual role in regulation of vulval morphogenesis such that it can positively as well as negatively regulate competence of VPCs. The fact that all three *Cbr-pry-1* alleles exhibit this defect suggests that this phenotype may not be a mere allele-specific defect.

Based on the cell morphology, the *Cbr-pry-1(sy5353)* mutants also display what seems to be a transformation of fate of the entire posterior set of Pn.p cells (P7.p- P11.p) towards that of the P12.pa cell. The fact that the P7.p and P8.p cells remain unfused strengthens the notion that these cells have adopted a non vulval fate not because they have assumed the fused cell fate but probably because they have taken up the fate of a different cell (possibly that of P12.pa.). To get a better understanding of this transformation, we studied the expression of gene expression markers such as *Cel-lip-1::gfp*, *Cel-egl-5::gfp* in *C. briggsae*. However, both markers did not express in their expected target cells. This seems to suggest a potential alteration in the regulation *Cel-lip-1*and *cel-egl-5* expression pattern in *C. briggsae*. Therefore, to get a better insight into this observation, future experiments could be aimed at generating reporter constructs that would reveal the expression pattern of *Cbr-lip-1* and *Cbr-egl-5* in the Pn.p cells in *C. briggsae*.

Nonetheless, these findings bring to light a unique defect with respect to disruption of *pry-1* function in *C. briggsae* that is not seen in *Cel-pry-1(mu38)* mutants. Taken together the results from the phenotypic analysis and the rescue experiment suggest a potential alteration in the function of the *pry-1* gene between *C. elegans* and *C. briggsae.*

Table 3.1: Heat shock rescue experiment (31 °C for 24 hrs)

Genetic background	Percentage Muv	Percentage non- Muv	N
Cbr-pry-1(sy5353); bhEx59[hs::Cbr-pry-1 + Cel-myo-2::gfp]	47.3%	52.7%	163

Table 3.2: Analysis of Q neuroblast migration defect in *Cbr-pry-1(sy5353)*) mutants

Strain	**Animals displaying QR.d posterior migration**	**N**
bhEx25[mec-7::gfp+myo-2::gfp]	2%	47
Cbr-pry-1(sy5353); bhEx25[mec-7::gfp+myo-2::gfp]	68.8%	45

Table 3.3: Vulval induction pattern of *Cbr-pry-1(sy5353)*, *Cel-pry-1(mu38)* mutants

Genotype	Muv penetrance	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	N
Wild-type AF16	0% (>100)	0%	0%	100%	100%	100%	0%	>100
***Cbr-pry-1* (sy5353)**	90% (50)	39%	82%	100%	100%	17%	21%	41
***Cel-pry-1* (mu38)**	37% (53)	19%	38%	100%	100%	90%	6%	31

Table 3.4: Analysis of *dlg-1::gfp* expression in *Cbr-pry-1(sy5353)* mutants

Strain	P7.p	P8.p	P9.p	N
Cbr-pry-1; *dlg-1::gfp*	63%	77.2%	27.2%	22

Figure.3.1: *Cel-pry-1* expression pattern during the early developmental stages of wild-type *C. briggsae*

(**A, B**) show the expression of *Cel-pry-1::gfp* in multiple cells in the wild type *C. briggsae* embryo just prior to hatching. (**C-F**) show *Cel-pry-1::gfp* being expressed in the some of the P cells (based on cell morphology) during the L1 larval stage.

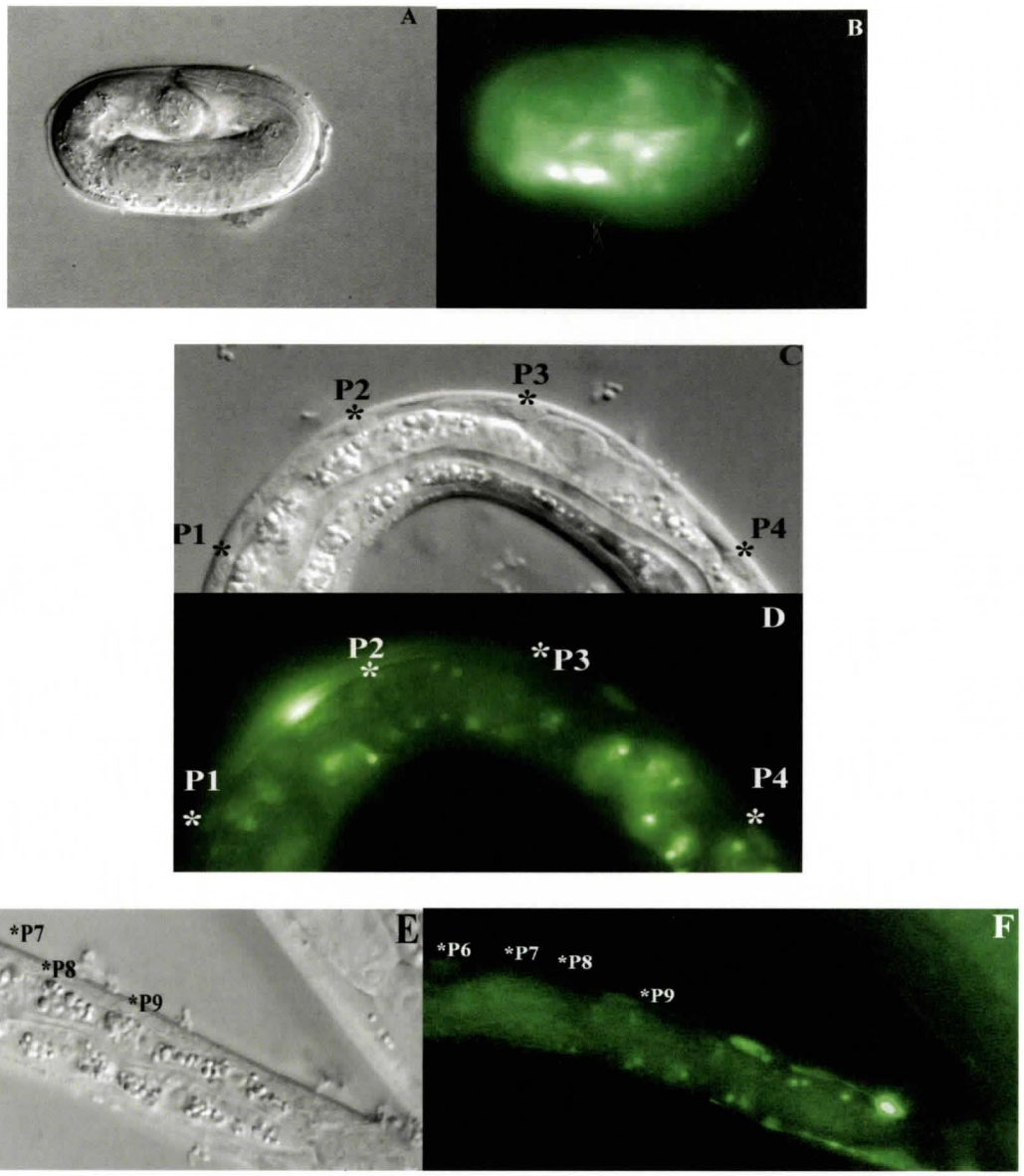

A
B
C
P2
P3
P1
P4
D
P2
*P3
P1
P4
E
*P6
*P7
*P8
*P9
F
*P6
*P7
*P8
*P9

Figure.3.2: *Cel-pry-1* expression pattern during *C. briggsae* vulval development

(**A, B**) show the observed *Cel-pry*-1 expression pattern during the early L3 larval stage at the time of vulval induction in wild-type *C. briggsae* animals. Here GFP expression is seen predominantly in the P6.p cell. The brightly fluorescing cell seen above P6.p is most likely to be the ventral uterine (VU) cell. (**C, D**) show the *Cel-pry-1::gfp* expression during the mid L4 larval stage where the expression is seen mainly among one of the descendents of P6.p namely the VulF cells. (**E, F**) show the expression of *Cel-pry-1* during the adult stage where the expression of *Cel-pry-1* continues to persist in the descendents of P6.p. The cells expressing GFP in (F) are most likely the VulFs.

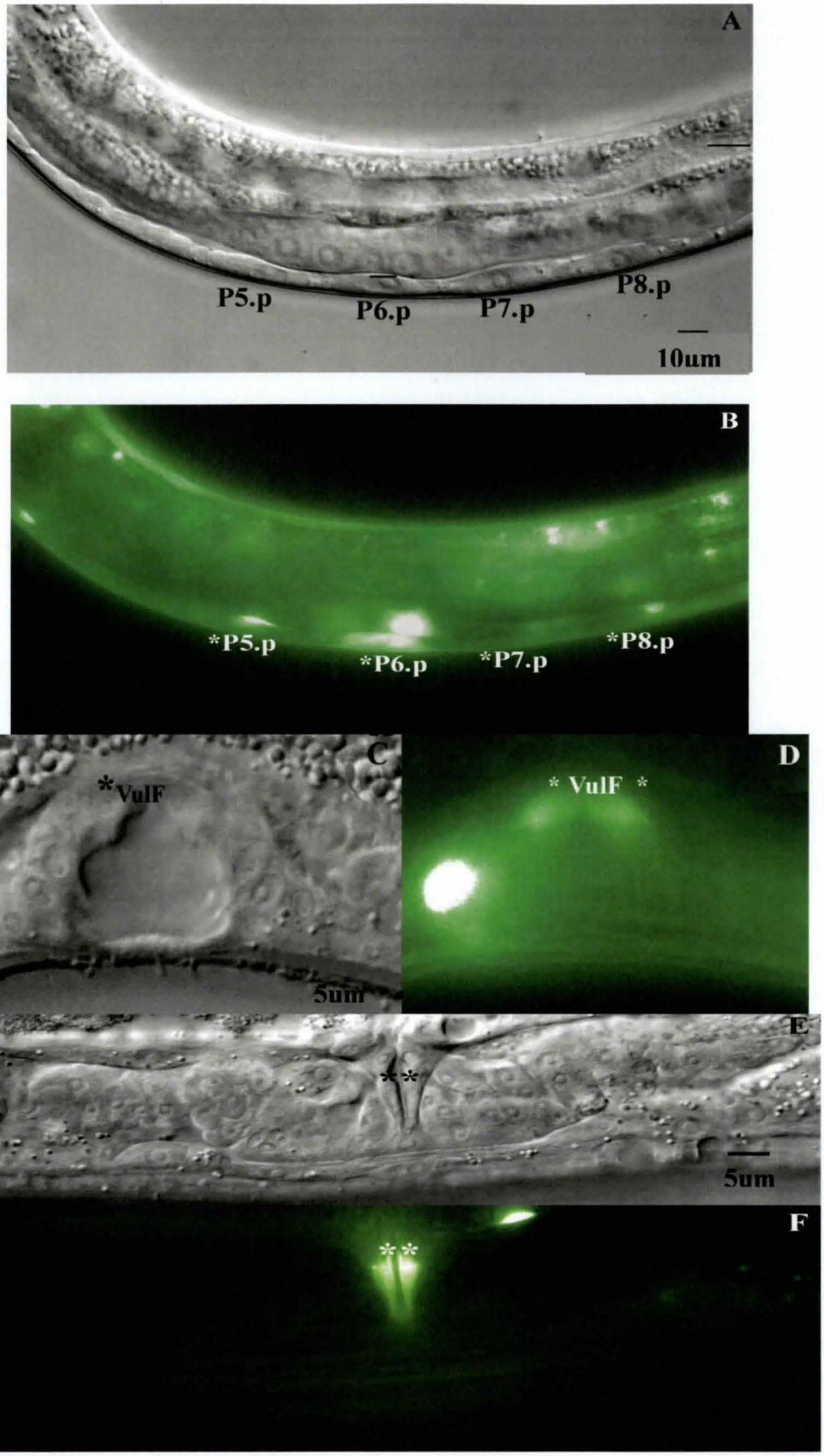

A
P5.p
P6.p
P7.p
P8.p
10um
B
*P5.p
*P6.p
*P7.p
*P8.p
C
*VulF
5um
D
* VulF *
E
**
5um
F
**

Figure.3.3: *Cel-pry-1* expression pattern in *Cbr-pry-1(sy5353)* mutants

(**A, B**) show the expression pattern of *Cel-pry-1::gfp* among the *Cbr-pry-1(sy5353)* mutants. The GFP expression is not seen among the ectopically induced anterior VPCs. The cells that show GFP expression (**denoted by ***) are most likely to be neuronal cells (based on their morphological features) found along the ventral hypodermis during the mid L4 larval stage. The neuronal cells generally are much smaller in size compared to those cells that adopt an epidermal fate. In addition, the neuronal cells also display a much smaller and denser nucleolus compared to those cells that adopt an epidermal cell fate.

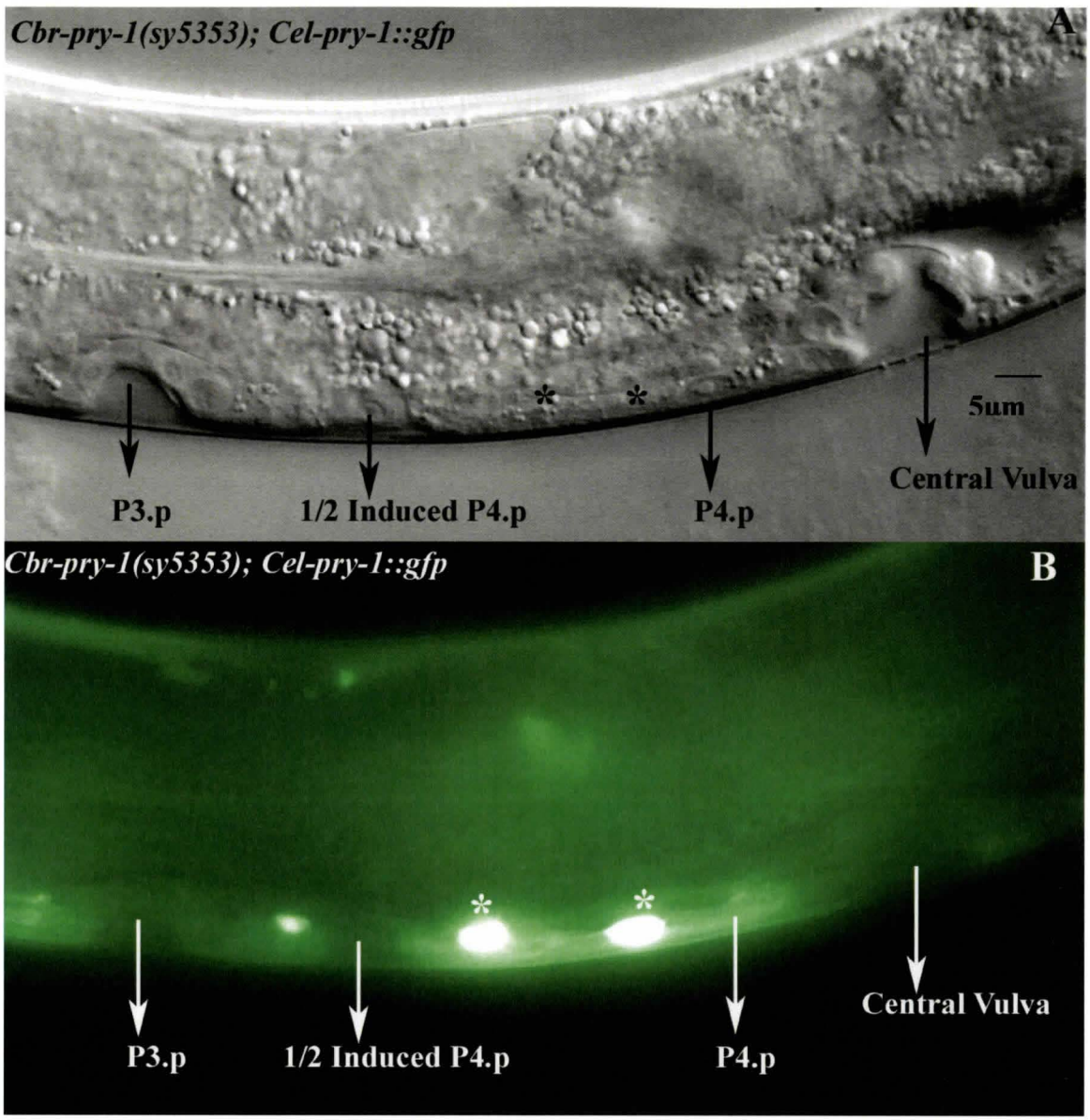

Cbr-pry-1(sy5353); Cel-pry-1::gfp
A
5um
P3.p
1/2 Induced P4.p
P4.p
Central Vulva
Cbr-pry-1(sy5353); Cel-pry-1::gfp
B
P3.p
1/2 Induced P4.p
P4.p
Central Vulva

Figure.3.4: Analysis of Q neuroblast migration pattern among wild-type and *Cbr-pry-1(sy5353)* mutants

(**A, B**) show the wild-type migration pattern of the QL and QR cells labeled PVM and AVM where AVM is seen along the anterior side while PVM migrates towards the posterior side of the animal. (**C, D**) show the altered pattern of migration in the *Cbr-pry-1(sy5353)* mutants where both AVM and PVM are seen on the posterior side of the animal, potentially due to a gain of function of *mab-5* in AVM.

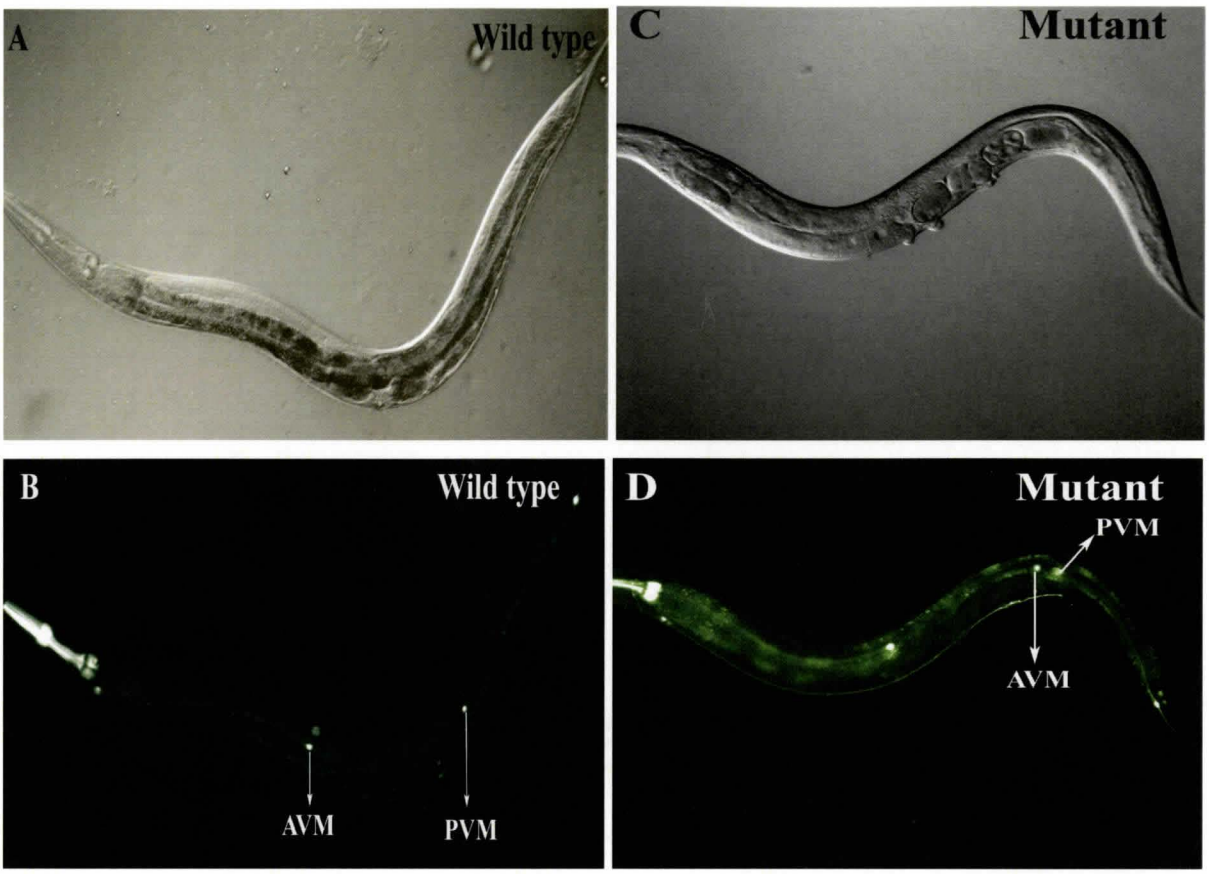

A
Wild type
C
Mutant
B
Wild type
AVM
PVM
D
Mutant
PVM
AVM

Figure.3.5: The vulval phenotype of *Cbr-pry-1(sy5353)* mutants

(A) In wild type *C. briggsae* the 3 VPCs P5.p- P7.p are always induced. (**B**) By contrast, in *Cbr-pry-1(sy5353)* mutants, the VPCs anterior to P6.p, namely P3.p, P4.p and P5.p frequently assume induced cell fates while those VPCs such as P7.p, P8.p which lie posterior to P6.p frequently adopt a non induced fate.

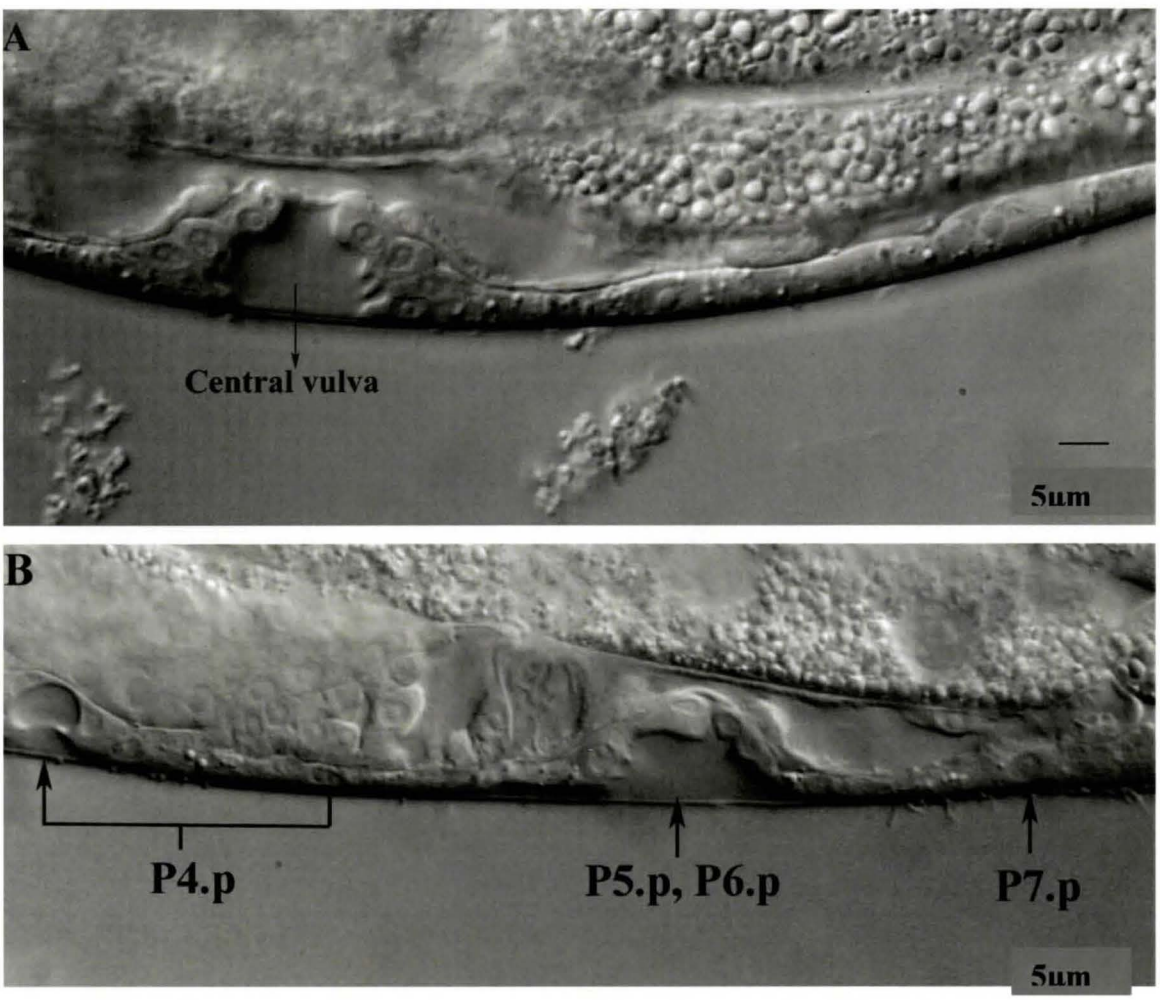
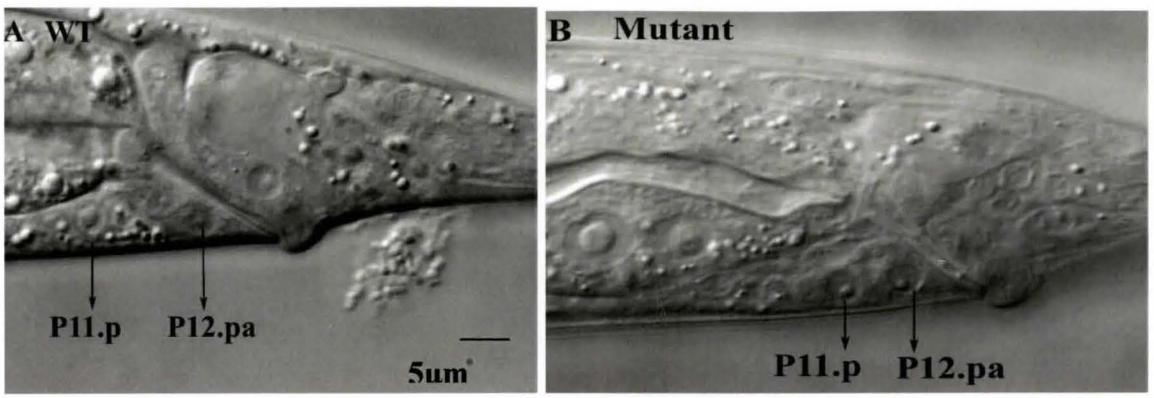
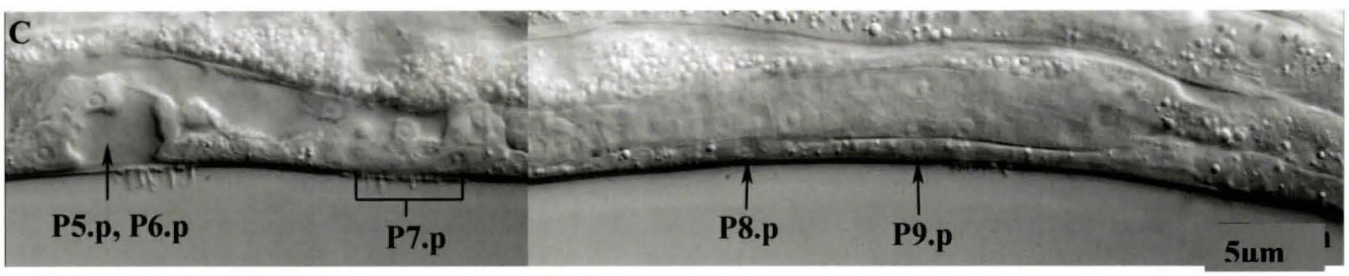

A
Central vulva
5um
B
P4.p
P5.p, P6.p
P7.p
5um

Figure.3.6: Fate transformation of the posterior Pn.p cells observed in *Cbr-pry-1(sy5353)* mutants

(**A, B**) highlight the transformation of fate of the P11.p cell towards that of the-P12.pa cell in the *Cbr-pry-1(sy5353)* mutants. (**C**) Shows the transformation of fate of the posterior set of Pn.p cells towards that of the P12.pa cell in the *Cbr-pry-1(sy5353)* mutants. The posterior Pn.p cells seen in the *Cbr-pry-1(sy5353)* mutants are much smaller in size compared to the wild type Pn.p cells and the size of the nucleolus in these cells in also much smaller than wild-type Pn.p cells. (**D**) Shows the morphology of the VPCs during the early L3 larval stage, prior to vulval induction in the *Cbr-pry-1(sy5353)* animals. The difference in the morphology of the P7.p and P8.p cells is clearly visible.


A WT
P11.p
P12.pa
5um
B Mutant
P11.p
P12.pa


C
P5.p, P6.p
P7.p
P8.p
P9.p
5um

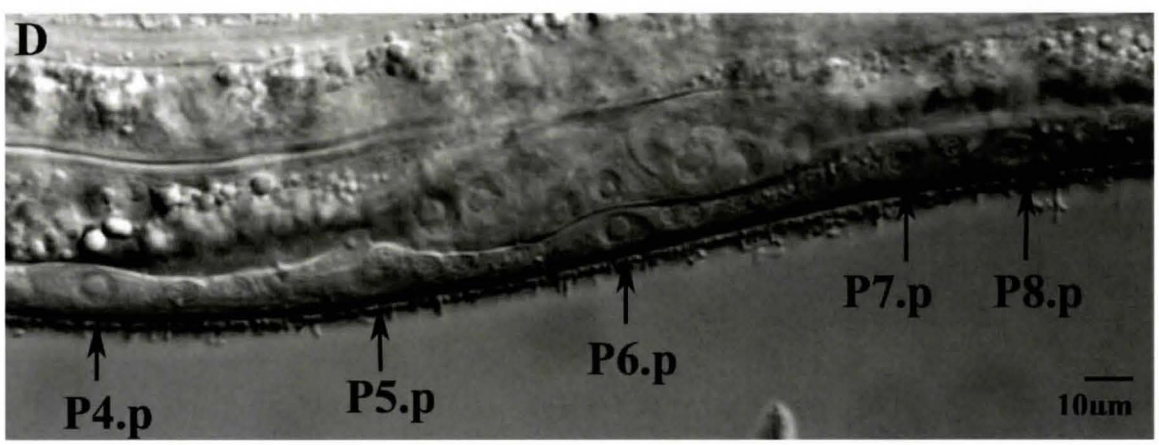

D
P4.p
P5.p
P6.p
P7.p
P8.p
10um

Figure.3.7: Analysis of *dlg-1::gfp* expression among wild type and *Cbr-pry-1(sy5353)* mutants

(A, B) show the wild-type expression pattern of *dlg-1::gfp* in the vulva. GFP expression is seen only among the unfused VPCs and not among those adopting non vulval fused fates. **(C, D)** show *dlg-1::gfp* expression in the P7.p and P8.p cells of the *Cbr-pry-1(sy5353)* mutants, showing that they exist as unfused cells.

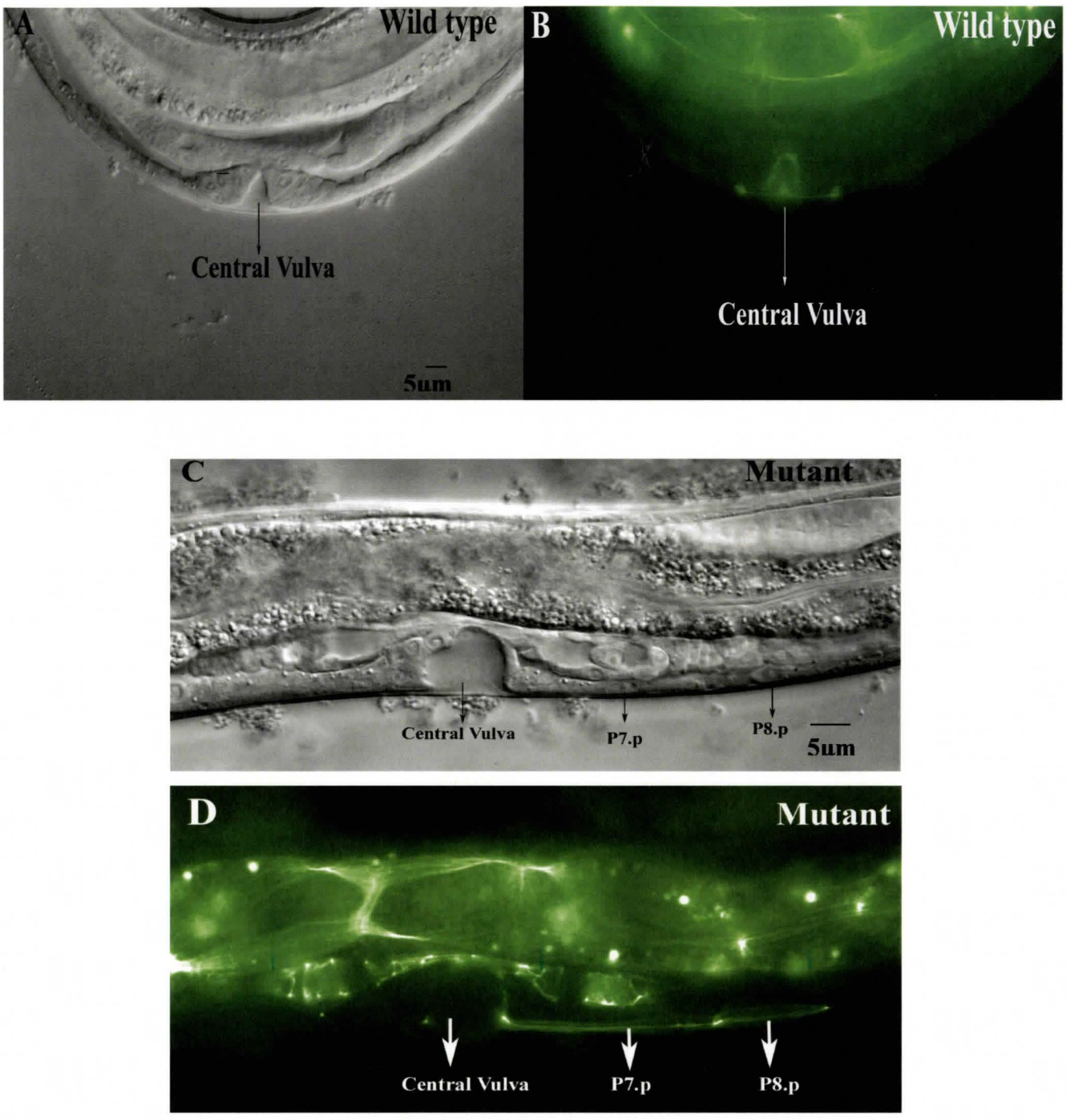
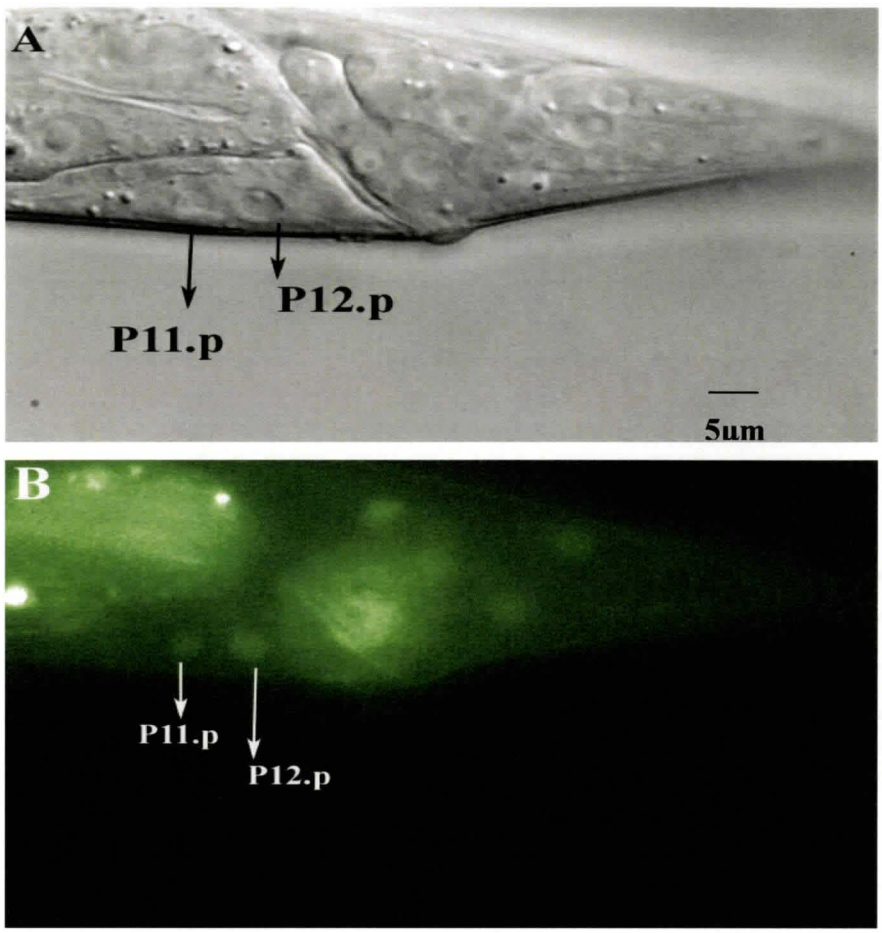

A
Wild type
Central Vulva
5um
B
Wild type
Central Vulva
C
Mutant
Central Vulva
P7.p
P8.p
5um
D
Mutant
Central Vulva
P7.p
P8.p

Figure.3.8: Aberrant expression of *Cel-lip-1::gfp* in *Cbr-pry-1(sy5353)* mutants

(**A, B**) show the expression of *Cel-lip-1::gfp* in P11.p as well as P12.pa cells.


A
P11.p
P12.p
5um
B
P11.p
P12.p

CHAPTER IV: Role of the canonical Wnt pathway components in *C. briggsae* vulval development

4.1 Introduction

A loss of function of *pry-1* in *C. elegans* causes an overinduced vulval phenotype, where VPCs other than P (5-7).p adopt 1° or 2° VPC fates. The fact that this phenotype is opposite to that observed in *bar-1* mutants, which display underinduced vulval phenotypes suggests that *pry-1/* Axin functions as a negative regulator of Wnt signaling during in vulval development (Eisenmann, 2005; Gleason et al., 2002). In a related experiment, a truncated version of *bar-1* that generates a protein lacking the amino-terminal GSK-3β phosphorylation site (ΔN-BAR-1), was found to result in ectopic induction of VPCs (Gleason et al., 2002). In addition, analysis of double mutants suggests that the overinduced vulval phenotype of *Cel-pry-1(mu38)* mutants is dependant upon *bar-1*, *pop-1* and *lin-39* (Gleason et al., 2002).

Interestingly, the overinduced phenotype caused by the *Cel-pry-1* mutation was not found to be dependent on the Ras pathway (Gleason et al. 2002). Mutations that strongly reduced Ras signaling were not found to affect the overinduced vulval phenotype caused by either a loss of *pry-1* function or stabilization of BAR-1. This suggests that the Wnt signaling pathway can directly affect the *lin-39* hox gene expression (Gleason et al., 2002).

The results from the phenotypic analysis and gene expression studies conducted on the *Cbr-pry-1(sy5353)* mutants reveal some interesting differences between what is seen in *C. elegans*. Given the nature of these differences, we were interested in determining whether this disparity in the phenotypes may be a consequence of an altered regulation of the canonical Wnt signal transduction pathway in *C. briggsae.* To get a better understanding of the *pry-1* function in *C. briggsae*, we wanted to investigate if the functioning of this gene was dependent on key regulators of the Wnt pathway such as *bar-1*(ß-catenin) and *pop-1(*TCF). Furthermore we also wanted to determine if the *Cbr-pry-1* function was dependent on key downstream targets of the Wnt pathway such as *lin-39, mab-5* in specification of vulval cell fates.

4.2 Results

4.2.1 Phenotypic analysis of *pop-1, bar-1* and *sys-1* in *C. briggsae* vulval development: We investigated the role of orthologous Wnt pathway components in *C. briggsae* using the RNA interference (RNAi) approach. RNAi refers to the process where a homologous double stranded RNA (dsRNA) is introduced specifically to target a gene's product to bring about the silencing of that gene (Fire et al., 1998).

Knocking down *Cbr-pop-1* function by RNAi was able to reduce the penetrance of the Muv phenotype of *Cbr-pry-1(sy5353)* from 90% to 36% (**Table 4.1, 4.2, Fig.4.1**). Interestingly, the *Cbr-pop-1 RNAi* in control animals induced a highly penetrent Vul phenotype (**Table 4.1, 4.2, Fig.4.1**). A similar phenotype is observed in the *C. elegans*

pop-1 RNAi-treated animals (**Table 4.1, 4.2**) or among viable *Cel-pop-1* alleles such as *hu9, q645* (Korswagen et al., 2002)

It is possible that another HMG box factor in *C. elegans* could be functioning redundantly with *pop-1* in the VPC fate specification process. To test this possibility, we knocked down *Cel-son-1* function by RNAi in *Cel-pop-1(q645)* loss of function mutants, to see whether this could induce a complete Vul phenotype similar to what was seen in *Cbr-pop-1*(RNAi) treated animals. *Cel-son-1* is a HMG1/2-like DNA-binding protein. Previous studies have shown that the disrupting the function of *Cel-son-1* by RNAi causes defects in several Wnt pathway mediated developmental processes such as vulval development, male spicule development, gonad development, etc. (Jiang and Sternberg, 1999). Knocking down *Cel-son-1by RNAi* did not induce a complete Vul phenotype in the *Cel-pop-1(q645)* mutants (**Table 4.1, 4.2**). However, the RNAi treated animals displayed a gonad defect very similar to that seen in *Cbr-pop-1(RNAi)* treated animals (**Fig.4.6**). It is also worth mentioning that the *Cel-son-1*(*RNAi*) treated control animals did not show any notable phenotype in the vulva or the gonad.

The RNAi-mediated knock down of *Cbr-bar-1* was able to bring about a reduction in the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants. The Muv penetrance in the *Cbr-bar-1(RNAi); Cbr-pry-1(sy5353)* was reduced from 90% to 29% (**Table 4.1, 4.2**). However, the *Cbr-bar-1* mediated suppression of the *Cbr-pry-1(sy5353*) Muv phenotype was variable, as on a separate occasion, the same experiment did not show a similar suppression (**Table 4.1, 4.2**). Moreover, in control animals, *Cbr-bar-1*(*RNAi*) did not

affect the vulval induction pattern but gave rise to a protruding vulva phenotype in adult animals. Similar experiments done in *C. elegans* have shown that knocking down *Cel-bar-1* and *Cel-pop-1* by means of RNAi were able to suppress the Muv phenotype of the *Cel-pry-1(mu38)* mutants efficiently (Gleason et al., 2002).

In addition to *Cbr-bar-1*, we also tested whether the β–catenin *Cbr-sys*-1 had any role in the development of the vulva. Previous studies conducted in *C. elegans* have shown that *sys-1* cooperates with the Wnt signaling pathway and genetically interacts with *pop-1* in establishing the proximal-distal axis of the gonad (Kidd et al., 2005; Siegfried et al., 2004). Knocking down *Cbr-sys-1* by RNAi did not cause any notable change to the *Cbr-pry-1(sy5353)* phenotype. However, the animals displayed defects in gonad development (**Table 4.1, 4.2; Fig.4.7**).

Thus these findings observed in *Cbr-pry-1(sy5353)* mutants suggest that the functioning of the Wnt signaling pathway in *C. briggsae* is mainly dependent on regulators of the Wnt pathway such as *bar-1*(β–catenin) and *pop*-1(TCF). However, the activities of some of the pathway components have diverged from *C. elegans*.

4.2.2 Phenotypic analysis of *lin-39* and *mab-5* in *C. briggsae* vulval development: The hox genes *lin-39* and *mab-5* are two well known targets of the canonical Wnt signaling pathway in *C. elegans* (Korswagen et al., 2002; Maloof and Kenyon, 1998). *lin-39* is a key determinant of the vulval equivalence group (Salser and Kenyon, 1992; Sternberg, 2005). *lin-39* is uniformly expressed in all VPCs prior to vulval induction (Maloof and

Kenyon, 1998). Loss of function of *lin-39* causes the presumptive VPCs to fuse with the underlying hyp7 cell (Salser et al., 1993). Previous studies have shown that Wnt signaling via BAR-1/β-catenin is necessary to maintain *lin-39* expression in the P (3-8).p cells (Gleason et al., 2002; Wagmaister et al., 2006). Moreover, the expression of *lin-39* is sufficient for vulval development to bypass the requirement for BAR-1 function in producing competent VPCs (Gleason et al., 2002). Previous studies have also shown that knocking down *lin-39* by RNAi can suppress the Muv phenotype of *Cel-pry-1(mu38)* mutants (Gleason et al., 2002).

The gene *mab-5* on the other hand seems to have an opposite role to *lin-39* in regulating VPC competence. Previous studies have shown that the competence of VPCs to adopt vulval fates correlates with their anterior-posterior position along the ventral midline (Sommer and Sternberg, 1994; Sommer and Sternberg, 1996). The P7.p and P8.p cells in particular are less sensitive to inductive signaling than P6.p (Clandinin et al., 1997). This difference in the sensitivity is attributed to the activity of *mab-5*, which is primarily expressed in P7.p and P8.p cells but not among the P (3-6).p cells (Salser et al., 1993). Moreover, analysis of gain of function mutants of *mab-5* reveals that the overexpression of *mab-5* in all the VPCs reduces the sensitivity of all VPCs to inductive signaling (Clandinin et al., 1997).

We wanted to determine if the ectopic induction of anterior VPCs P3.p and P4.p in the *Cbr-pry-1(sy5353)* mutants was caused by elevated levels of *Cbr-lin-39* activity and likewise whether the loss of competence among the posterior VPCs P7.p and P8.p cells

was caused by elevated levels of *Cbr-mab-5* activity in those cells. Knocking down *Cbr-lin-39* by RNAi reduced the Muv penetrence of the *Cb-pry-1(sy5353)* mutants from 90% to 3.7 % (**Fig.4.2; Table 4.1, 4.2**). Knocking down *Cbr-mab-5* however did not enhance the competence of the posterior VPCs (**Table 4.1, 4.2**). A lack of any scorable phenotype raised the question of whether or not the *Cbr-mab-5 RNAi* construct was working. To resolve this issue, *Cbr-mab-5(RNAi)* was carried out in *Cbr-pry-1(sy5353)* mutants expressing *Cel-mec-7::gfp*. Loss of function of *mab-5* in *C. elegans* causes the anterior migration of the QL.d (Korswagen et al., 2002). We wanted to investigate whether knocking down *Cbr-mab-5* can bring about a similar effect this in the *Cbr-pry-1(sy5353)* mutants. The *Cbr-mab-5(RNAi)* did not induce the anterior migration of the QL.d (**Table.4.3**). However, it was still able to suppress the posterior migration of the QR.d, a defect associated with gain of function of *mab-5*, seen among both among *Cel-pry-1(mu38)* as well as *Cbr-pry-1(sy5353)* mutants [refer previous chapter section 3.1.3.2]. Furthermore, the male progeny arising from the *Cbr-mab-5(RNAi)* treated control animals were all found to have severe abnormalities associated with the tail development, a hallmark feature associated with loss of function of *mab-5* in *C. elegans* (**Fig.4.8**).

These observations suggest that the *Cbr-mab-5 RNAi* construct is indeed functional. However, it could be also that the RNAi knockdown of *Cbr-mab-5* was insufficient to confer competence to the posterior VPCs P7.p and P8.p. Taken together, these results suggest that the ectopic inductions of anterior VPCs are is dependent on the activity of *Cbr-lin-39* while the loss of competence among posterior VPCs may not be dependent on activity of *Cbr-mab-5*.

4.2.3 Genetic interactions between *Cbr-lin-12* and *Cbr-pry-1*: Pervious work by B. Nagagireesh from our lab showed that *Cbr-egl-17::gfp*, a secondary cell lineage marker (Gupta and Sternberg, 2003), was predominantly being expressed in the progeny of ectopically induced anterior VPCs of the *Cbr-pry-1(sy5353)* mutants, suggesting that they had adopted 2° fates. As LIN-12/Notch pathway is a key regulator of the 2° cell fate specification in *C. elegans* (Sternberg, 2005), we wanted to test its role in arbitrating the effect of the overactivated Wnt signaling in VPCs.

If the anterior Muv phenotype of the *Cbr-pry-1(sy5353)* mutants is a consequence of elevated LIN-12/Notch signaling, then knocking down *Cbr-lin-12* should suppress the Muv penetrance of the *Cbr-pry-1(sy5353)* mutants. However, knocking down *Cbr-lin-12* by RNAi, did not suppress the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants (**Fig.4.3, Table 4.1, 4.2**). The *Cbr-lin-12(RNAi)* in control animals did not cause any notable defect in the vulval induction pattern but affected the vulval morphology (**Fig.4.3C**). These results suggested that the LIN-12/Notch pathway may not have a direct role in mediating the specification of the 2° cell fates among the ectopically induced VPCs or may indeed function in parallel to the Wnt pathway in *C. briggsae* to specify the secondary cell fates among anterior VPCs. It is worth mentioning here that although knocking down *Cbr-lin-12* by means of RNAi did not efficiently suppress the Muv phenotype of the mutants, it nonetheless enhanced the level of competence among the posterior VPCs (**Fig.4.3**). The RNAi treated *Cbr-pry-1(sy5353)* mutants displayed a normal central vulva where P5.p, P6.p and P7.p cells were fully induced. Furthermore, the morphology of P8.p was very similar to that of the wild type animals.

On the other hand, knocking down *Cel-lin-12* by RNAi in *C. elegans* was able to suppress the multivulva phenotype of *Cel-pry-1(mu38)* mutants efficiently (**Table 4.1, 4.2**). This suggests that specification of the 2° cell fates among the ectopically induced VPCs in *C. elegans* might be dependent on activity of the LIN-12/Notch pathway. The *Cel-lin-12(RNAi)* in control animals did not induce any defect in neither the vulval induction nor the vulval morphology (**Table 4.1, 4.2**). However, these control animals displayed much a slower growth compared to the wild type. In addition, they also appeared to be sick.

Previous work by B. Nagagireesh showed that the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants is independent of the gonad derived signal. To resolve the issue of whether the LIN-12/Notch pathway has any role in mediating the specification of secondary cell fates among ectopically induced VPCs, gonad ablation experiments (which involves destroyed by means of a laser beam) were carried out in *egl-17::gfp* expressing *Cbr-pry-1(sy5353)* mutants. This would completely eliminate the source of inductive signaling by the Ras pathway, and thus should also effectively eliminate the lateral signaling acting among the VPCs mediated by the LIN-12/Notch pathway.

We found that the ectopically induced VPCs as well as P6.p in these gonad ablated animals expressed *Cbr-egl-17::gfp* (**Fig.4.4, Table 4.4**) thereby demonstrating that the VPCs were still capable of adopting secondary cell fates in the absence of both inductive and Notch signaling activity. To be more certain about this inference, the same experiment was repeated in *Cbr-pry-1(sy5353)* mutants expressing the primary cell

lineage marker *Cbr-zmp-1::gfp* (Gupta et al., 2003), which is expressed mainly in Vul E cells in *C. briggsae*. Interestingly we did not observe any GFP expression among the ectopically induced VPCs in this genetic background (**Fig.4.4, Table 4.4**). This result strongly suggests that the Wnt signaling pathway in *C. briggsae* is solely capable of specifying secondary cell fates among VPCs. However the possibility that the Wnt pathway is independently activating the *lin-12*/Notch signaling cannot be ruled out.

4.2.4 Genetic interaction between *Cel-pop-1* and *Cel-lin-12*: The results from the *Cel-lin-12* (RNAi) experiment mentioned earlier coupled with those obtained from the gonad ablation experiments done in *C. briggsae*, suggest that specification of the 2° cell fates by the Wnt pathway might depend on LIN-12/Notch signaling in *C. elegans* but not in *C. briggsae*. Thus to study this further a *Cel-pop-1* RNAi experiment was performed in a *Cel- lin-12(n952)* Gain-of- function background.

The *Cel-lin-12(n952)* mutants display a characteristic Muv phenotype since all the VPCs adopt 2° cell fates (Greenwald and Seydoux, 1990) (**Fig.4.5**). It should be mentioned that the vulval induction caused in this mutant background takes place solely under the influence of the LIN-12 pathway. This is because a gain of function of LIN-12/Notch causes both Z1.ppp and Z4.aaa cells (found in the developing gonad in the L1 larval stage) to adopt the fate of a Ventral Uterine (VU) cell as opposed to one of them assuming the fate of the Anchor Cell (AC), as seen in wild-type animals (Greenwald and Seydoux, 1990).

Knocking down *Cel-pop-1* function by RNAi was able to fully suppress the Muv phenotype in these animals. The suppression was so potent that it rendered a vast majority of the animals Vulvaless (**Fig.4.5, Table 4.1, 4.2**). The *Cel-pop-1(RNAi)* in control animals did not have any effect on the vulval inductions but affected the vulval morphology. This result suggests that in *C. elegans, pop-1* is epistatic to LIN-12/Notch pathway with respect to specification of the 2° cell fate among VPCs. However, these results should also be verified by means of a genetic approach to further validate the inference from this experiment to that in *C. elegans.*

4.3 Discussion

In this chapter, I described experiments to examine the role of some of the key regulators of the Wnt pathway such as the β-catenin encoding genes *Cbr-sys-1*(β-catenin), *Cbr-bar-1*(β-catenin) and the TCF /LEF factor *Cbr-pop-1* by means of an RNAi strategy. Knocking down *Cbr-sys-1* by RNAi did not cause any notable change to the *Cbr-pry-1(sy5353)* phenotype. On the other hand knocking down *Cbr-bar-1* was significantly able to suppress the Muv phenotype of the *Cbr-pry-1(sy5353)* animals. Similarly knocking down *Cbr-pop-1* was also able to efficiently suppress the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants. These results demonstrate that the vulval phenotype of *Cbr-pry-1(sy5353)* mutants is dependent on *Cbr-bar-1* and *Cbr-pop-1.*

Interestingly, knocking down *Cbr-pop-1* in *C. briggsae* control animals resulted in a highly penetrent Vul phenotype. This was surprising since no such vulval induction defect is observed in *Cel-pop-1(RNAi)* treated animals or among viable *Cel-pop-1* alleles

such as *hu9, q645*. Although *Cel-pop-1(RNAi)* is able to suppress the Muv phenotype of the *Cel-pry-1(mu38)* animals, it nonetheless does not result in a Vul phenotype. This suggests that *pop-1* function may be required mainly among the P3.p, P4.p and P8.p cells to promote cell proliferation in *C. elegans* whereas in *C. briggsae, pop-1* function may be necessary in all VPCs. In light of this finding, we considered the possibility that in *C. elegans* another HMG box factor in addition to *pop-1* may function redundantly in the vulval induction process. To test this, we knocked down the HMG box factor *Cel-son-1* by RNAi in a viable allele of *Cel-pop-1(q645)* to check if this can induce a complete Vul phenotype. The *Cel-son-1* RNAi however did not bring about a complete Vul phenotype in any of the animals. However, the *Cel-son-1(RNAi)* enhanced the gonad defect of the *Cel-pop-1(q645)* mutants. This suggests that *Cel-son-1* may not be involved in the vulval induction process but may have a role in the regulation of the gonad development. It is possible that there may be other HMG box genes in *C. elegans*, which might have a role in regulating vulval induction.

Having identified a divergence in the functioning of *Cbr-pop-1,* it was important to examine how this change affects downstream gene targets. Previous studies in *C. elegans* have shown *lin-39* and *mab-5* to be direct targets of the Wnt signaling pathway. Furthermore, it is known that *Cel-lin-39* has a positive role in assigning competence to the VPCs while *mab-5* activity decreases the sensitivity of the posterior VPCs such as P7.p and P8.p to the inductive signal by potentially negatively regulating *Cel-lin-39* (Clandinin et al., 1997; Sternberg, 2005). It is conceivable that ectopic induction of anterior VPCs in *Cbr-pry-1(sy5353)* animals is caused by elevated levels of *Cbr-lin-39*

activity while loss of competence in the P7.p and P8.p cells results from elevated levels of *Cbr-mab-5* activity.

The role of *Cbr-lin-39* in regulating the fates of anterior VPCs is supported by my results. However, knocking down *Cbr-mab-5* did not in anyway alter the competence of the posterior VPCs. A previous study that examined the vulval phenotype of a *lin-39(n709)* reduction of function mutant shows that the posterior VPCs P7.p and P8.p frequently adopt non vulval fused fate in these mutants potentially due to a gain of function of *mab-5* in those cells (Clandinin et al., 1997). However, the P7.p and P8.p cells in the *Cbr-pry-1(sy5353)* mutants often remain unfused and resemble the P12.pa cell in morphology. These observations coupled with the result of the *Cbr-mab-5(RNAi)* seem to suggest that *mab-5* may not have a role in regulating competence of the posterior VPCs. Recent studies conducted in *P. pacificus*, have revealed that *Ppa-lin-39* function is not required for the process of assigning competence to VPCs and hence has acquired very different roles compared to *Cel-lin-39*. Similarly mutations *in Ppa-mab-5* have been found to cause ectopic vulval inductions of P8.p, while no such phenotype is seen in *C. elegans* (Sommer R.J, 2005; Zheng et al., 2005). In the context of comparing *C. elegans* and *C. briggsae*, it appears that while the role of *lin-39* in vulval development is conserved between the two species, the role of *mab-5* may not be conserved between *C. elegans* and *C. briggsae*.

An earlier observation by B. Nagagireesh from our lab showed that the ectopically induced VPCs were adopting 2° cell fates. Given that the LIN-12/ Notch pathway plays a major role in the specification of 2° cell fates (Sternberg), I examined its contribution to

the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants. Knocking down *Cbr-lin-12* by *RNAi* in *Cbr-pry-1(sy5353)* mutants did not suppress the Muv phenotype. However, the level of competence among the posterior VPCs, P7.p, and P8.p in the *Cbr-lin-12(RNAi)* treated mutant animals was greatly enhanced. This suggests a possible inhibitory role for the LIN-12/Notch pathway in conferring competence among the posterior VPCs in *C. briggsae*. Taken together the result from this experiment suggests that the LIN-12/Notch pathway may not have a direct role to play in the specification of the 2° cell fates among the ectopically induced VPCs or it may function in parallel to the Wnt pathway in *C. briggsae* to specify the secondary cell fates among anterior VPCs.

Alternately, It is possible that the *Cbr-lin-2(RNAi)* did in fact suppress the ectopic anterior pseudovulvae from adopting 2° cell fates but the Muv phenotype continued to persist probably because the ectopically induced VPCs had adopted the primary fate. To resolve this issue, gonad ablation experiments were performed (which in effect should eliminate the source of inductive and lateral signaling acting among the VPCs) in the *Cbr-egl-17::gfp* and *Cbr--zmp-1::gfp* expressing *Cbr-pry-1(sy5353)* mutants. Interestingly, while expression of *Cbr-egl-17::gfp* was observed, no sign of *Cbr--zmp-1::gfp* expression was seen in the ectopic vulval inductions in the gonad ablated *Cbr-pry-1(sy5353)* animals. This clearly demonstrates that the VPCs that were induced had indeed adopted the secondary cell fate and that the Wnt signaling pathway is solely capable of inducing the 2° cell fates among the ectopically induced VPCs in *C. briggsae*.

On the other hand, specification of the 2° cell fates among the ectopically induced VPCs in *C. elegans* seems to be dependent on activity of the LIN-12/Notch signaling, as knocking down *Cel-lin-12* by RNAi in the *Cel-pry-1(mu38)* mutants was efficiently able to suppress the Muv phenotype of the *Cel-pry-1(mu38)* mutants. To deepen our understanding of the interaction between the Wnt and the Notch signaling pathways in *C. elegans*, we tested the effect on knocking down *Cel-pop-1* in a *Cel-lin-12(n952)* gain of function background. Knocking down *Cel-pop-1* by RNAi fully suppressed the Muv phenotype of the *Cel-lin-12(n952)* mutants, giving rise to a Vul phenotype. This finding indicates that the LIN-12/Notch pathway may function upstream of the Wnt signaling pathway in *C. elegans* in the specification of secondary cell fates among the VPCs.

However, it should be mentioned that the result from this experiment is not conclusive. It is important to verify these results by means of a genetic approach before coming to a conclusion. One experiment that would shed more light on the nature of the genetic interaction between the Wnt and the Notch pathway would be to examine the phenotype of the *Cel-lin-12(n952)* mutants in the background of a *Cel-pop-1* null mutation. The viable *Cel-pop-1(hu9)* loss of function allele offers an ideal genetic background for such a study. If the results from the genetic experiment further validate the result of the RNAi experiment mentioned above, one can then proceed to investigate into the molecular nature of such an interaction between the Wnt and the Notch pathways.

In summary, the findings from my Masters research project brings to light a novel role for the Wnt signaling pathway in *C. briggsae* vulval development where it has evolved to

positively as well as negatively regulate vulval precursor competence. In addition to the divergence of the molecular mechanisms controlling the competence of VPCs our study also shows that the ability to confer 2° fates on VPCs mediated by the Wnt signaling pathway is conserved between *C. elegans* and *C. briggsae*. Taken together, the results from this present study show that the although the vulval morphology between *C. elegans* and *C. briggsae* is indistinguishable, the genetic control governing its development has diverged, by way of Wnt signaling function evolving towards simultaneous activation and repression of vulval cell competence in different VPCs based on their location on the ventral hypodermis.

4.4 Conclusion

The phenomenon where the P(4-8).p cells form a vulva equivalence group which give rise to the 3°-2°-1°-2°-3° fate pattern is something that is unique to the Rhabditidae family (Sommer, 2005). In this regard, *C. elegans* is itself is something of an exception in that P3.p is also a member of the equivalence group in about 50% of the individuals. Furthermore, the non-vulval cells such as P1.p- P3.p on the anterior side and P9.p –P11.p on the posterior side, usually fuse with the surrounding hypodermal syncitium. The Diplogastridae vulval pattern on the other hand, as seen in the case of the nematode *Pristionchus pacificus* and all other species of the Diplogastridae family, the non-vulval cells in the anterior and posterior body region are eliminated via programmed cell death. The vulva in these nematodes is comprised of the P (5-7).p cells with a 2°-1°-2° cell fate pattern (Sommer, 2005). Comparative studies of vulval development between different

nematode species have revealed evolutionary variability, mostly at the level of vulva induction. However, it is interesting to see that despite the variability in cell induction and lineages, the final vulval cell fate pattern seems to be surprisingly conserved between most species.

Thus with respect to *C. elegans* and *C. briggsae*, future work on Wnt targets should offer more insights into the extent of conservation and divergence in Wnt signaling-mediated cell fate specification and how alterations in gene expression and interactions are compensated to give rise to seemingly identical tissue morphologies.

Table 4.1: RNAi knockdown experiments

Gene tested by RNAi	Genotype	Vulval phenotype					N
		WT	Complete Vul	Partial Vul	Muv	Pvul	
Cbr-bar-1 Round I	+; *mfIs42[Cel-sid-2+myo-2::dsRED]*	68.9%	---	---	---	31%	29
	Cbr-pry-1(sy5353); mfIs42	4%	---	---	96%	---	25
Cbr-bar-1 Round II	+; *mfIs4*	66.7%	---	---	---	33.3%	12
	Cbr-pry-1(sy5353): *mfIs42*	47%	---	23.5%	29.4%	---	17
Cbr-pop-1	+; *mfIs42*	8.8%	91.1%	---	---	---	45
	Cbr-pry-1(sy5353); *mfIs42*	39.5%	14.5%	10.5%	35.4%	---	48
Cel-pop-1	+	100%*	---	---	---	---	20
	lin-12(gain of function)	5%	80%	10%	5%	---	20
Cbr-lin-39	+; *mfIs42*	35.7%	---	64.3%	---	---	14
	Cbr-pry-1(sy5353); *mfIs42*	---	---	97.3%	3.7%	---	27
Cbr-lin-12	+; *mfIs42*	66.6%	---	---	---	33.3%	30
	Cbr-pry-1(sy5353); *mfIs42*	11.4%	---	14.2%	74.3%	---	29
Cel-lin-12	+	100%	---	---	---	---	12
	Cel-pry-1(mu38)	78.5%	---	11.9%	9.5%	---	42
Cel-son-1	+	100%	---	---	---	---	14
	Cel-pop-1(q645)	66.7%	---	33.3%	---	---	9
Cbr-sys-1	+; *mfIs42*	81.8%	---	18.1%	---	---	11
	Cbr-pry-1(sy5353); *mfIs42*	3.7%	---	3.7%	92.5%	---	27
Cbr-mab-5	*Cbr-pry-1(sy5353)*; *mfIs42*	---	---	3.4%	96.5%	---	29

*Animals showing abnormal vulval morphology

Table 4.2. Vulval induction patterns observed among RNAi treated animals

Gene tested by RNAi	Genotype	Vulval Induction pattern					
		P3.p	**P4.p**	**P5.p**	**P6.p**	**P7.p**	**P8.p**
Cbr-bar-1 Round I N=16	+; *mfIs42[Cel-sid-2+myo-2::dsRED]*	0%	0%	100%	100%	100%	0%
N= 29	*Cbr-pry-1(sy5353)*; mfIs42	51.7%	44.8%	100%	100%	100%	62%
Cbr-bar-1 Round II, N= 12	+; *mfIs4*	0%	0%	100%	100%	100%	0%
N= 17	*Cbr-pry-1(sy5353)*: *mfIs42*	5.8%	23.5%	100%	100%	100%	5.8%
Cbr-pop-1 N= 45	+; *mfIs42*	0%	0%	6.6%	6.6%	6.6%	0%
N= 48	*Cbr-pry-1(sy5353)*; *mfIs42*	12.5%	25%	83.3%	85.4%	68.7%	6.2%
Cel-pop-1 N= 12	+	0%	0%	100%	100%	100%	0%
N= 20	*lin-12*(gain of function)	5%	5%	10%	20%	5%	5%
Cbr-lin-39 N = 14	+; *mfIs42*	0%	0%	35.7%	92.8%	35.7%	0%
N= 27	*Cbr-pry-1(sy5353)*; *mfIs42*	0%	3.7%	0%	92.5%	3.7%	0%
Cbr-lin-12 N = 30	+; *mfIs42*	0%	0%	100%	100%	100%	0%
N=35	*Cbr-pry-1(sy5353)*; *mfIs42*	62.8%	71.4%	100%	100%	85.7%	8.5%
Cel-lin-12 N= 12	+	0%	0%	100%	100%	100%	0%
N= 30	*Cel-pry-1(mu38)*	3.3%	3.3%	100%	100%	93.3%	3.3%
Cel-son-1 N=14	+	0%	0%	100%	100%	100%	0%
N=9	*Cel-pop-1(q645)*	0%	0%	66.6%	100%	66.6%	0%
Cbr-sys-1 N = 11	+; *mfIs42*	0%	0%	81.8%	100%	81.8%	0%
N= 27	*Cbr-pry-1(sy5353)*; *mfIs42*	77.7%	70.3%	100%	100%	59.2%	22.2%
Cbr-mab-5 N= 29	*Cbr-pry-1(sy5353)*; *mfIs42*	72.1%	75.8%	100%	100%	27%	31%

Table 4.3: *Cbr-mab-5* (RNAi)

Strain	**QL.d Anterior migration**	**QR.d posterior migration**	**wild-type QL.d, QR.d migration**	**N**
bhEx25[*mec-7::gfp+myo-2::gfp*]	9.4%	---	90.6%	53
*Cbr-pry-1(sy5353)**; bhEx25[*mec-7::gfp+myo-2::gfp]*	2.17%	23%	74.8%	45

Table 4.4: Vulval induction pattern among gonad ablated *Cbr-pry-1(sy5353)* Animals

Genetic Background	**Vulval Induction pattern**					
	P3.p	**P4.p**	**P5.p**	**P6.p**	**P7.p**	**P8.p**
Cbr-pry-1(sy5353); Cel-egl-17::gfp N = 8	50%	37.5%	50%	100%	0%	0%
Cbr-pry-1(sy5353); Cel-zmp-1::gfp * N = 10	ND	ND	ND	ND	ND	ND

* The Muv penetrence in these animals was found to be 100%

Figure.4.1: *Cbr-pop-1*(RNAi) in wild-type and in *Cbr-pry-1*(sy5353) animals

(**A**) shows the normal induction and morphology of the vulva among wild-type *C. briggsae* animals. (**B**) Shows the complete Vul phenotype that is seen among control animals treated with *Cbr-pop-1(RNAi)*. These animals in addition to the Vul phenotype, also display a severely deformed gonad, seen as a ball of cells above the VPCs. (**C**) shows the suppression of the Muv phenotype among the *Cbr-pry-1(sy5353)* mutants treated with *Cbr-pop-1(RNAi)*.

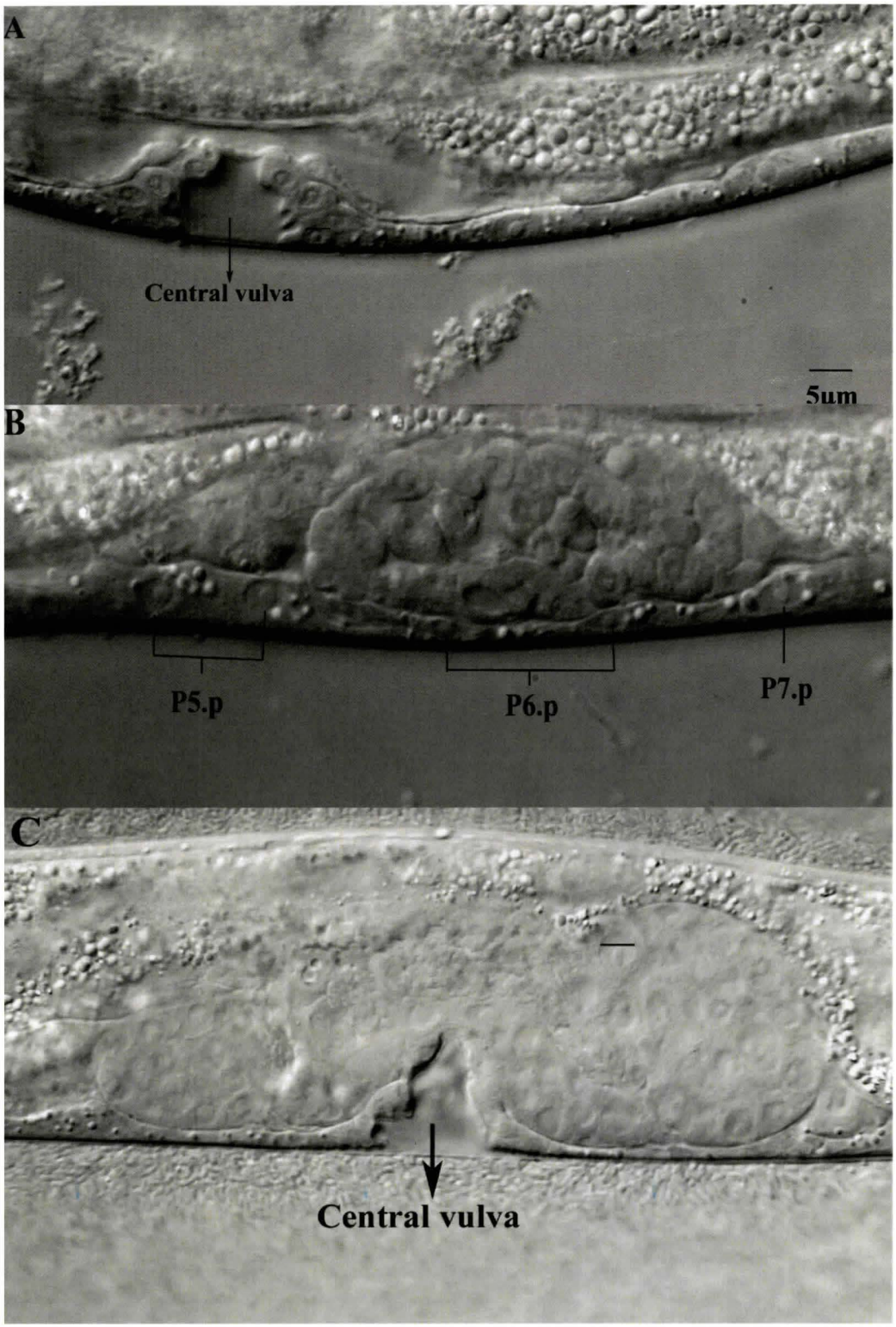

A
Central vulva
5um
B
P5.p
P6.p
P7.p
C
Central vulva

Figure.4.2: *Cbr-lin-39(RNAi)* in *Cbr-pry-1(sy5353)* animals

(**A, B**) show the suppression of the Muv phenotype of *the Cbr-pry-1(sy5353)* mutants when treated with *Cbr-lin-39(RNAi)*. Seen in both (**A, B**), are the invaginations produced by the sole induction of P6.p. P5.p and P7.p are uninduced.

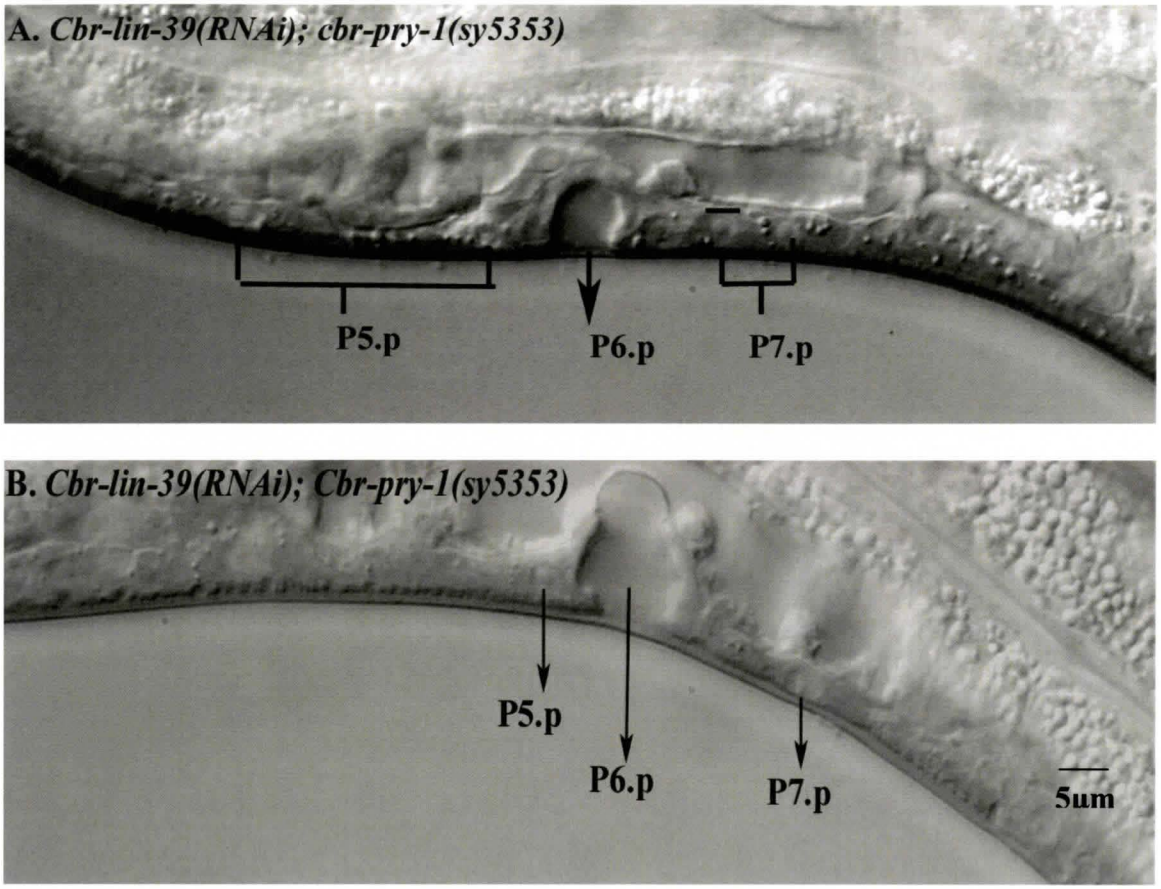
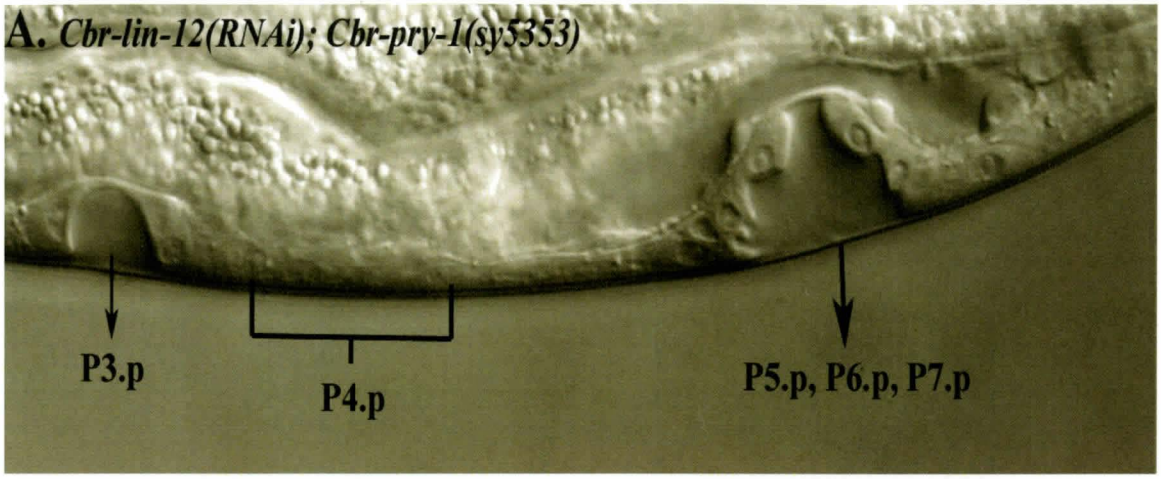
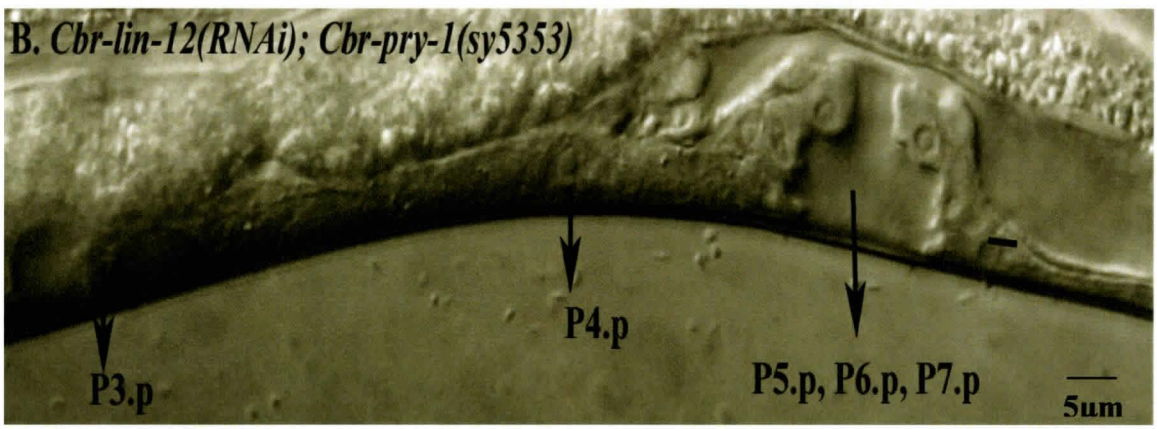

A. Cbr-lin-39(RNAi); cbr-pry-1(sy5353)
P5.p
P6.p
P7.p
B. Cbr-lin-39(RNAi); Cbr-pry-1(sy5353)
P5.p
P6.p
P7.p
5um

Figure.4.3: *Cbr-lin-12(RNA*i) in wild type, *Cbr-pry*-1(sy5353) mutants.

(A, B) show the apparent lack of suppression of the Muv phenotype of the *Cbr-pry-1(sy5353)* mutants when treated with *Cbr-lin-12(RNAi*). The presence of a WT looking central vulva shows that in addition to P5.p, P6.p being induced, P7.p has been fully induced in these animals, thereby demonstrating competence to take up vulval cell fates. (**C**) Shows the abnormal vulval induction pattern observed in *Cbr-lin-12(RNAi)* treated control animals.


A. Cbr-lin-12(RNAi); Cbr-pry-1(sy5353)
P3.p
P4.p
P5.p, P6.p, P7.p


B. Cbr-lin-12(RNAi); Cbr-pry-1(sy5353)
P4.p
P3.p
P5.p, P6.p, P7.p
5um

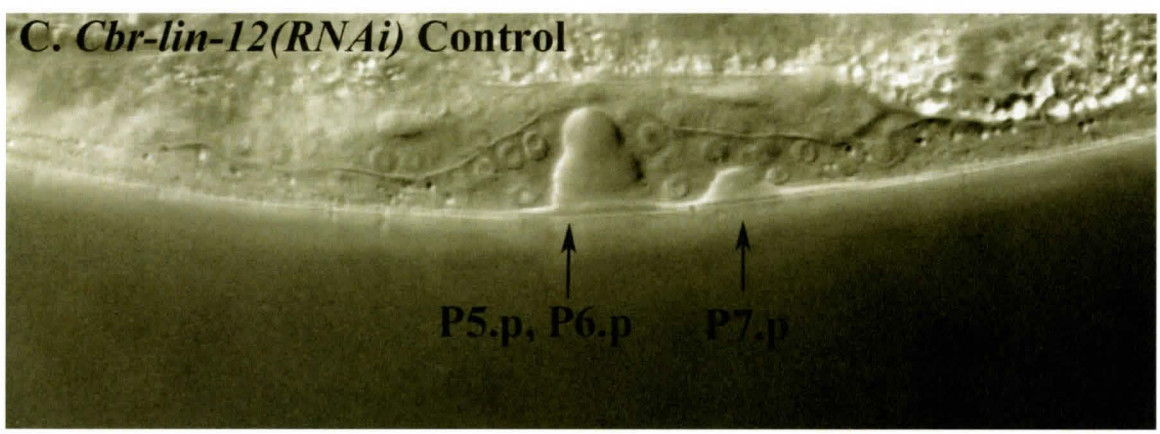

C. Cbr-lin-12(RNAi) Control
P5.p, P6.p
P7.p

Figure.4.4: Gonad ablation studies conducted in *Cbr-pry-1(sy5353)* animals

(**A, B**) highlight the fact that the VPCs in the *Cbr-pry-1(sy5353)* mutants are capable of adopting vulval cell fates even in the absence of the gonad dependent inductive and the laterally mediated LIN-12/Notch signaling pathways. Expression of secondary cell fate marker, *Cbr-egl-17::gfp* among the induced VPCs seen, suggests that these VPCs have adopted the secondary cell fate. (**C, D**) highlight the fact that in the absence of the inductive and lateral signaling pathways the induced VPCs are competent only adopt secondary cell fates and not the primary cell fate, as seen by the lack of *Cbr-zmp-1::gfp* reporter expression among the induced VPCs (Which are most likely to be P4.p*, P6.p, denoted by arrow sign) the *Cbr-pry-1(sy5353)* mutants.

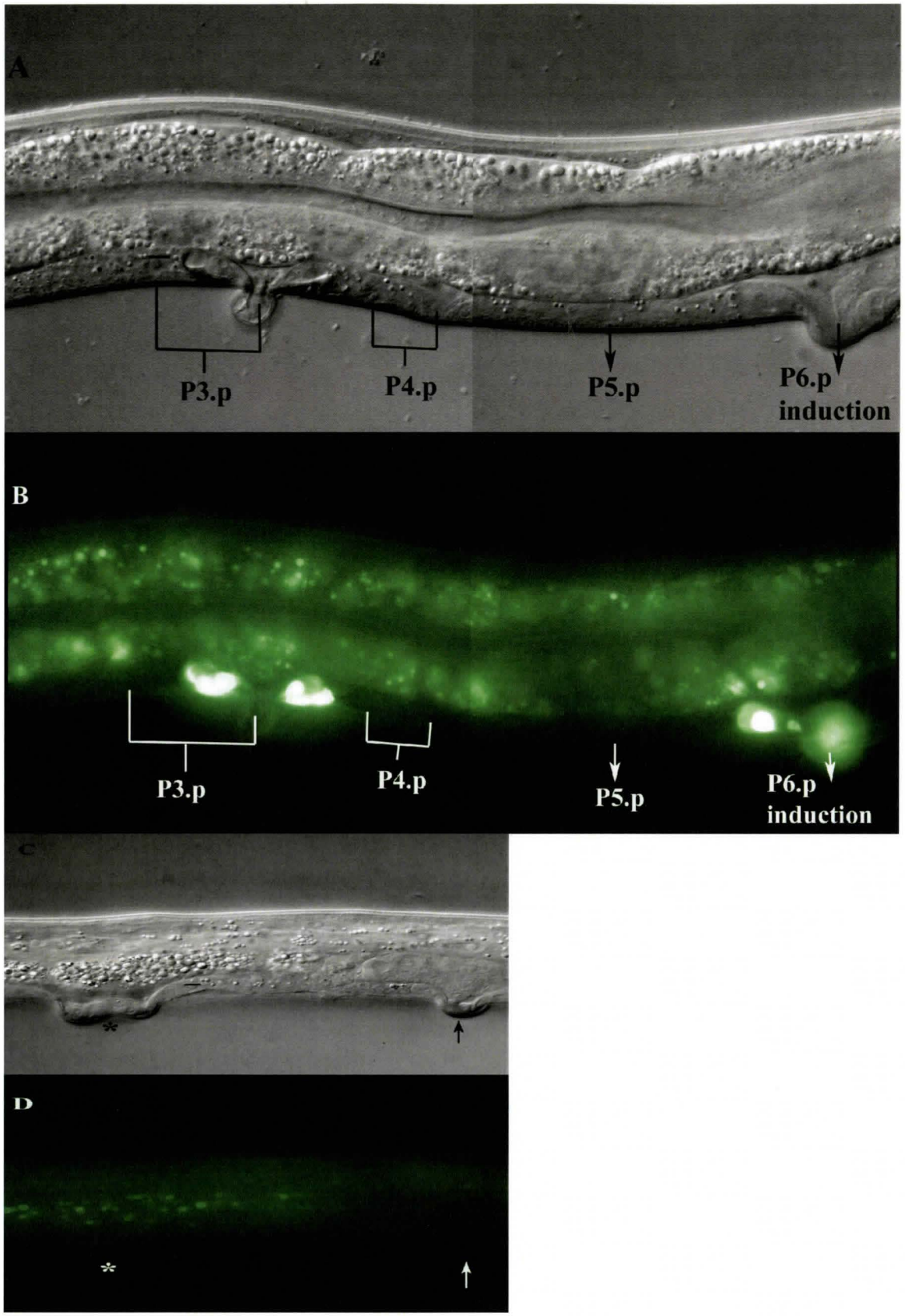
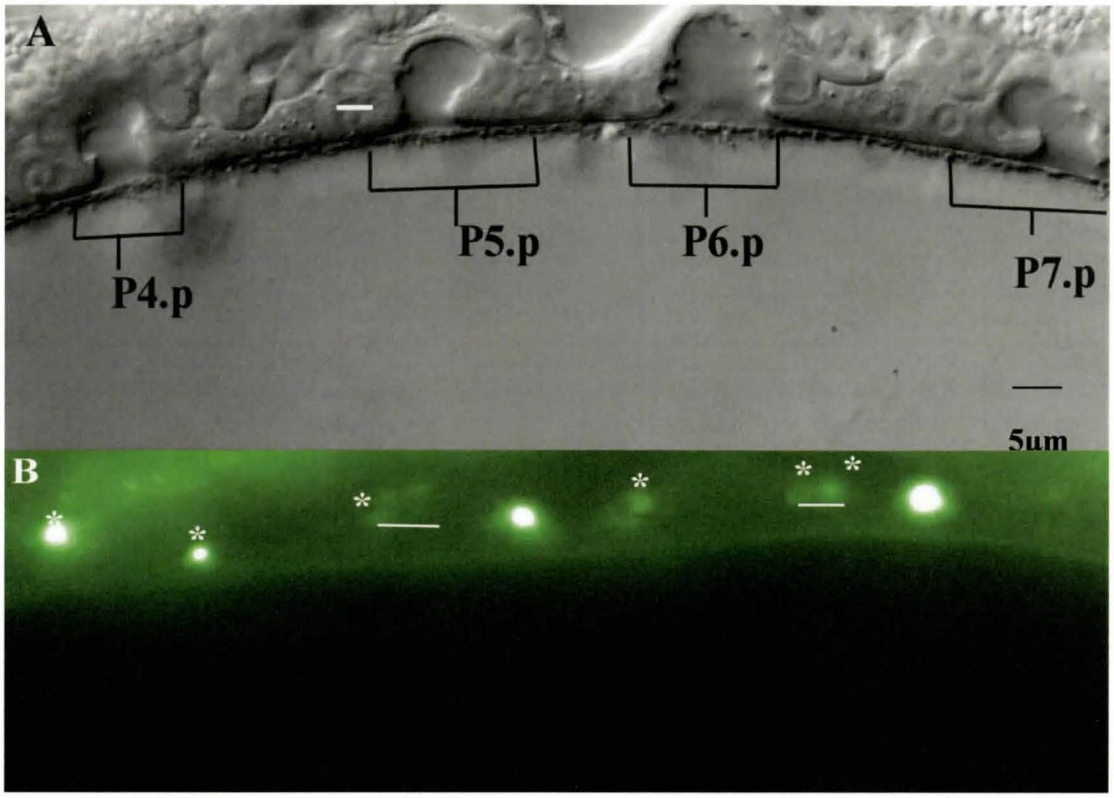

A
P3.p
P4.p
P5.p
P6.p
induction
B
P3.p
P4.p
P5.p
P6.p
induction
C
*
D
*

Figure.4.5: *Cel-pop-1(RNAi)* in *Cel-lin-12(n952)* gain-of-function mutants

(A, B) highlight the vulval induction and cell fate patterns seen among *Cel-lin-12(n952)* gain of function mutants. The expression of *Cel-lin-11::gfp* reporter in the induced VPCs suggests that they had adopted the secondary cell fate (Gupta el al. 2003). (**C**) Shows the suppression of the Muv phenotype of the *Cel-lin-12(n952)* gain of function mutants when treated with *Cel-pop-1(RNAi)*. Seen in (**C**) is the uninduced VPCs P5.p- P7.p.


A
P4.p
P5.p
P6.p
P7.p
5µm
B

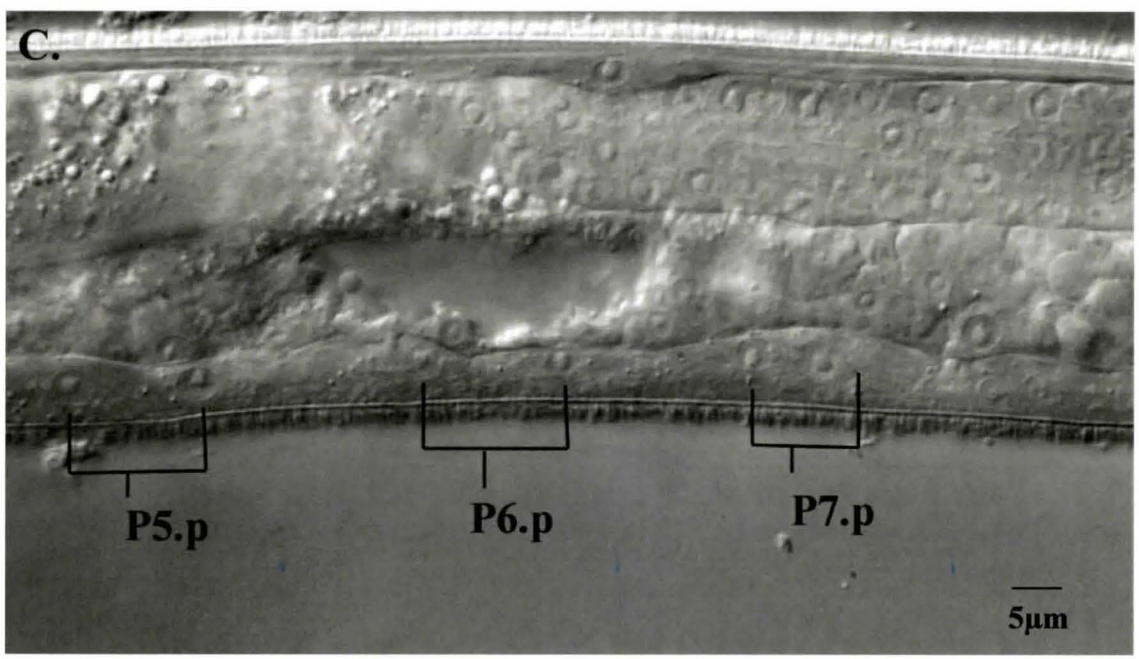

C.
P5.p
P6.p
P7.p
5µm

Figure.4.6: *Cel-son-1(RNAi)* in *Cel-pop-1(q645)* mutants

(**A**) Shows the normal wild-type L4 stage vulva. (**B**) Shows the vulval induction pattern and gonad defect observed in *Cel-pop-1(q645)* animals treated with *Cel-son-1(RNAi)*.

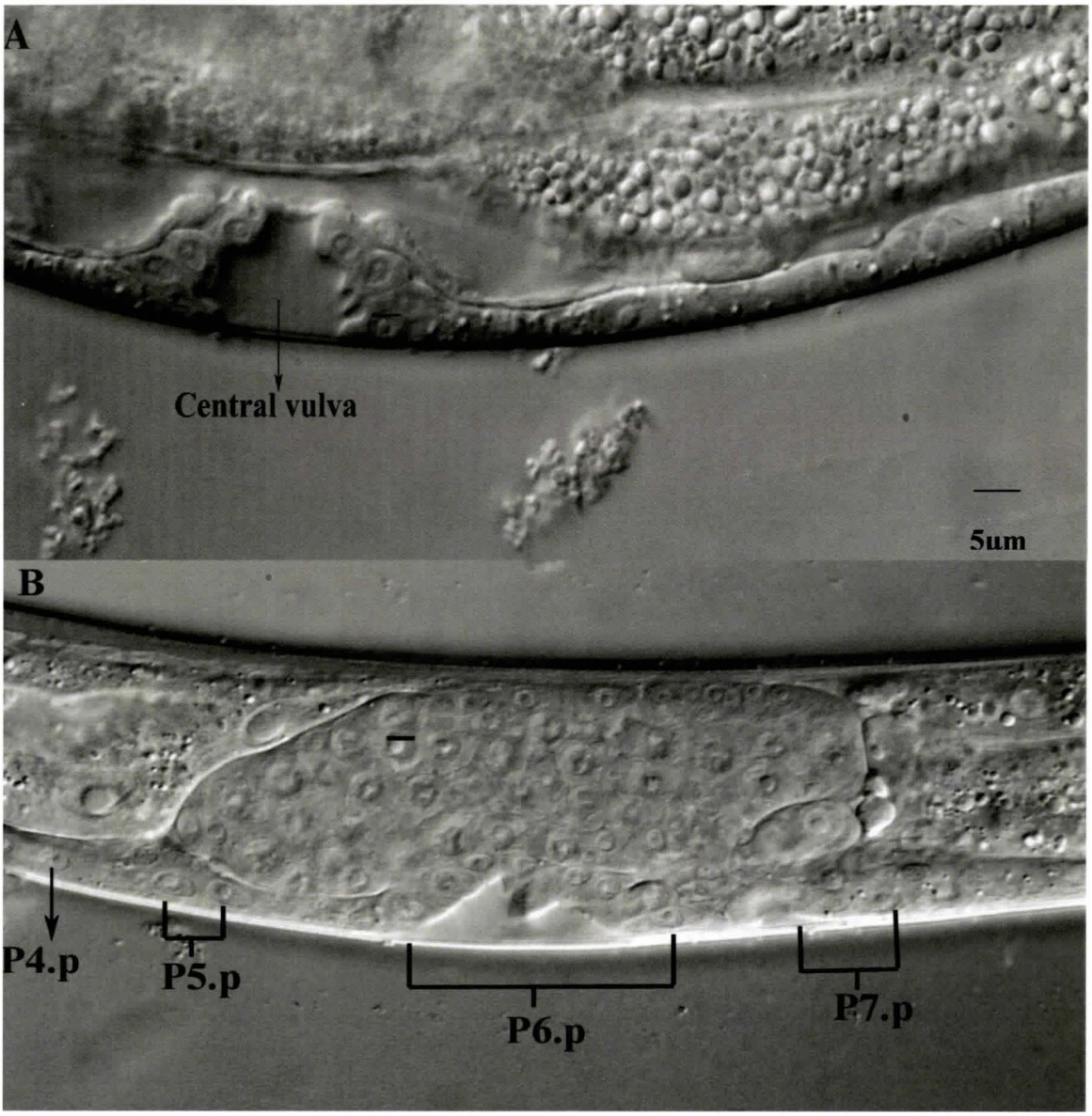

A
Central vulva
5um
B
P4.p
P5.p
P6.p
P7.p

Figure.4.7: *Cbr-sys-1(RNAi)* in *C. briggsae*

(**A**) shows the normal induction and morphology of the vulva among wild-type animals. (**B**) Shows a folding defect in the posterior gonad arm of control animals treated with *Cbr-sys-1(RNAi)*. (**C**) Shows the vulval induction pattern and gonad defect observed in *Cbr-pry-1(sy5353)* mutants treated with *Cbr-sys-1(RNAi)*.

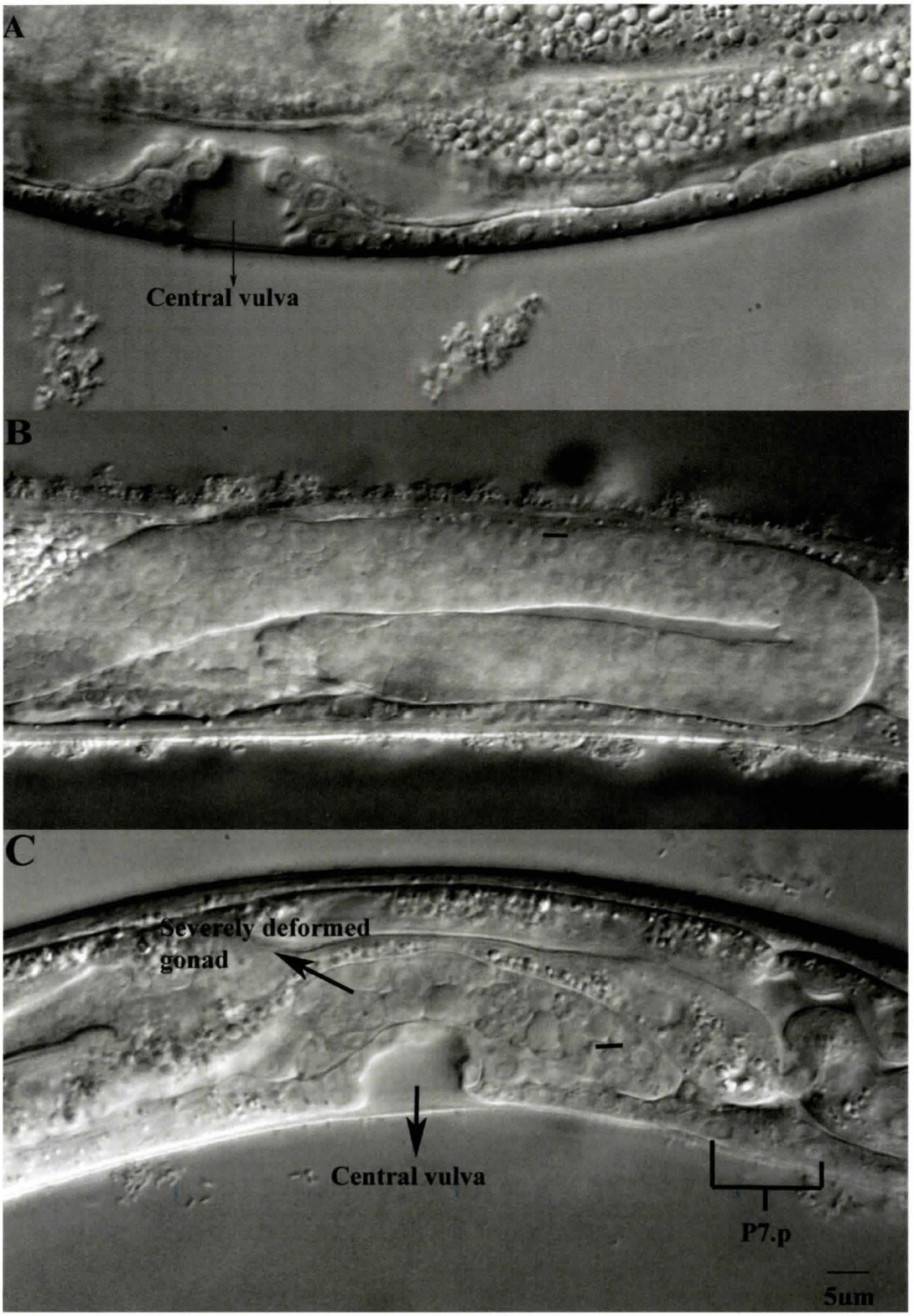
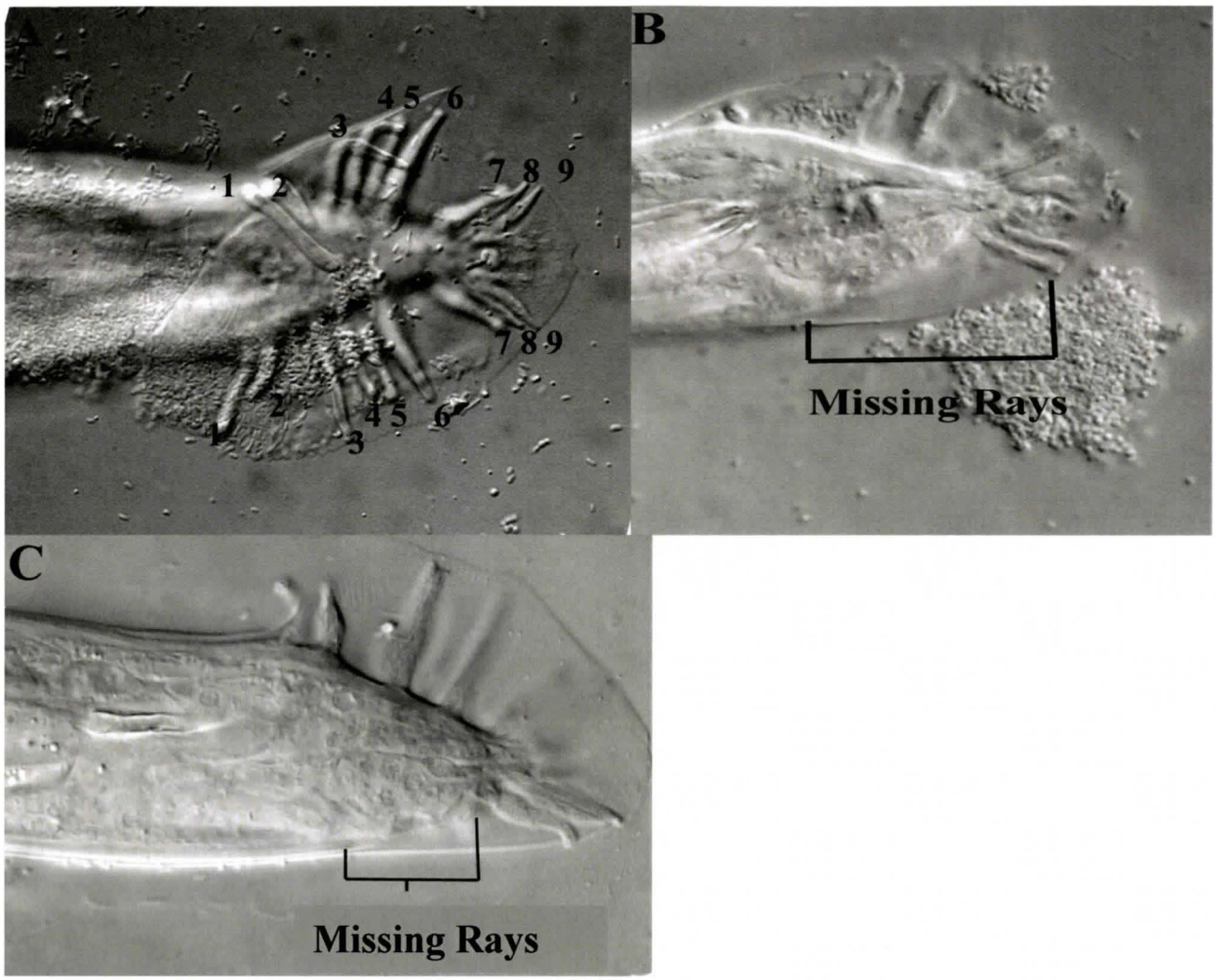

A
Central vulva
B
C
Severely deformed
gonad
Central vulva
P7.p
5um

Figure.4.8: Abnormal male tail development induced by *Cbr-mab-5* RNAi in wild type control animals

(A) shows the organization of the sensory rays on the tail of a typical wild type *C. briggsae* male. There are generally 9 bilateral pairs of rays on either side (L/R) of the tail. **(B)** Shows the absence of several rays in the mail tail of the wild type *C. briggsae* animals treated with *Cbr-mab-5(RNAi).*


A
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
B
Missing Rays
C
Missing Rays

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