Genetic Mapping of the M77 Short Pharynx Phenotype in *C. elegans*

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Abstract

Embryogenesis is a fascinating process during which a single cell transforms into a multi-cellular hybrid of organs and tissues. Our lab investigates the underlying genetic framework that drives organogenesis through our study of the model organism, Caenorhabditis elegans (*C. elegans*). *C. elegans* is a free-living nematode that has a pronounced pharynx, which is ideal for studying organogenesis from the incipience to the end of differentiation and morphogenesis. We canspecifically locate the mutation responsible for producing a short pharynx phenotype observed in the mutant line of C. elegans called M77, which is larval lethal. We described the mutant pharyngeal phenotype through light microscopy, immunocytochemisty, and we utilized complementation tests and genetic mapping to identify the location of the mutant gene, each procedure proving some explanation for a possible mechanistic pathway for morphogenesis. We also aimed to genetically balance the M77 strain. We were able to narrow down the location of the mutant gene to chromosome III between -3.10 and -4.47 mu that effectively puts the mor-1 gene, which was previously thought to be the possible gene causing the M77 phenotype, outside this mu range. However, we were not able to find any gene that exhibits phenotypes similar to the M77 mutation gene within the range of -3.10 and -4.47 mu. We believe that it might be a gene that has not been previously described and therefore we might be the first ones to describe this gene. We also successfully balanced the M77 strain. In addition, we observed that a 7% ethanol treatment suppressed larval death and the mutant worms progressed even through the L1 developmental stage. Our future goals include determining the identity of the M77 mutant gene through further complementation analyses, interval mapping, sequencing, and conducting a confirmation of the mutant gene identity through a transgenic rescue of the mutant worms. Moreover, we hope to determine the molecular pathway through which the M77 gene functions.

Introduction

Developmental biology is the study of processes and natural phenomena that occur during an organism's growth and differentiation. It is primarily concerned with the period of "becoming" rather than the period of "being" (Gilbert, 2006). More specifically, developmental biology seeks to understand the fascinating transformation of a single cell into a multi-cellular organism composed of tissues and organs. Generically, in virtually all multi-cellular organisms, the process of development begins with the fusion of an egg with a sperm, a process called fertilization. Fertilization occurs to yield a diploid zygote that matures to subdivide, specialize, and produce a multi-cellular organism such as a Homo sapiens (Gilbert, 2006; Ferrier, 2009; Charron, 2010). Developmental biology research is dedicated to understanding the causes behind numerous congenital diseases, such as Holt-Oram, which is an abnormality of the upper limbs and heart (Fan et al., 2003; Mori & Bruneau, 2004). Moreover, it has been key in shaping our current understanding of cancer. Developmental biologists are taking steps to identify signaling pathways that control tissue growth and organization (Edwards, 1999). Developmental research has elucidated the vital importance of the period known as organogenesis for the proper development of an organism. Moreover, it has highlighted the potential risks of certain irregularities that may occur during the period of embryogenesis. These risks may result in major birth disorders, such as Holt-Oram syndrome (Fan, Liu, & Wang, 2003; Mori & Bruneau, 2004).

It is the focus of this senior thesis project to understand the genetic mechanisms and regulatory machinery governing and conducting the transformation of a single cell into a functional multi-cellular organ. The purpose of this research is to expand the findings of the previous research conducted by Andrew Ferrier by using the fore-gut (pharynx) of the microscopic nematode, Caenorhabditis elegans (*C. elegans*), as a model to further genetically map and specifically locate the mutation responsible for producing the short pharynx phenotype observed in the mutant line of *C. elegans* called M77 with enough precision to identify its chromosomal location. The pharynx is not unique to C. elegans. It is found in humans as well; the pharynx is part of the vertebrate alimentary canal and it extends to the larynx (Daniels, 2007; Tortora & Nielsen, 2009). *C. elegans* have an elongated, cylindrical pharynx that has a terminal bulb at one end. This research seeks to determine the genetic and molecular cause of the short pharynx phenotype resulting from a mutation in M77 worms. Simultaneously, we are aiming to preserve the mutated strain by genetically balancing the M77 allele with a deletion strain.

C. elegans As A Model Organism

Utilization of a model organism in biological research to study and understand a particular phenomenon and then, apply the learned knowledge to other organisms is vast and immensely common. The use of model organisms is largely possible because the metabolic and developmental mechanisms and pathways that exist today have largely evolved from a common point (Barr, 2003; Kaletta & Hengartner, 2006). Therefore, various organisms can be studied to better understand pathways that have been conserved over time. The usage of a model organism in biological research makes it easier for scientists to conduct research to explore and access the root causes of certain human diseases, which otherwise would require human experimentation (Barr, 2003; Kaletta & Hengartner, 2006). Human experimentation is not a viable research option because it requires the knowing consent of the subjects and depending on the research topic, the use of human subjects might be unethical. Moreover, because embryonic development occurs in the uterus, human experimentation limits the methods that researchers can use to investigate a topic (Rutstein, 1969).

In developmental biology, there are several model organisms that have been extensively used and studied. These studies have provided us with valuable information about the genetic and molecular pathways coordinating development. Already developed model organisms include

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chicks, sea urchins, Drosophila melanogaster, mice, Xenopus laevis, zebrafish, and C. elegans (Barr, 2003; Gilbert, 2006; Kaletta & Hengartner, 2006). In our study, we used C. elegans as our model organism for various reasons.

There are ample advantages in using any of the model organism listed above. However, the use of model organisms such as sea urchins and Drosophila pose certain disadvantages (Gilbert, 2006). Sea urchins are quite difficult to cultivate and manage in laboratory conditions beyond a certain stage. Drosophila undergoes a complicated developmental process, which makes the research problem more difficult to assess and solve (Gilbert, 2006). In fact, the complicity of Drosophila's developmental process drove the Nobel-Prize winner Sydney Brenner and his colleague to spearhead another search in the quest of finding a simple model organism (Gilbert, 2006). C. elegans is a simple model that overcomes the disadvantages of aforementioned model organisms (Gilbert, 2006). It enables the researchers to identify each gene in the C. elegans' genome and trace the lineage of each C. elegans' cell if desired.

C. elegans are about a millimeter long (Brenner, 1974; Gilbert, 2006). They are small, free-living, non-parasitic, soil nematodes that were introduced as a model organism in 1974 by Sydney Brenner, "The genetics of Caenorhabditis elegans," to study neurobiology and developmental biology. C. elegans is one of the simplest multicellular eukaryote that has recently gained popularity in the scientific community. It is being used to study various insightful biological processes, such as apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, aging and sex determination (Kaletta & Hengartner, 2006). In fact, programmed cell death, which is an evolutionarily conserved process used by multicellular organisms to eliminate unwanted cells, has been extensively studied in C. elegans (Adams & Cory, 1998; Conradt & Horvitz, 1998; Jacobson, Weil, & Raff, 1997; Metzstein, Stanfield, & Horvitz, 1998; Spector, Desnoyers, Hoeppner, & Hengartner, 1997). According to Spector and his colleagues, mammalian Bcl-2 family members might control apoptosis in an analogous way as CED- 9 in C. elegans (Spector et al., 1997).

Similarly, many other studies have revealed a remarkable biological similarity between C. elegans and other mammals. In fact, humans share numerous biological properties with C. elegans that have conserved and preserved various intact mammalian biological processes, such as program cell death or apoptosis (Metzstein et al., 1998). Consequently, scientists have taken advantage of this fact and conducted various research studies on C. elegans. The studies have led to innumerable, crucial discoveries not only in the field of biology but also in the medical field. For example, the first presenilin discovery was made through a research study on C. elegans in 1993. Presenilin has been identified as a component of γ-secretase complex, which is a key Alzheimer's disease's target (De Strooper et al., 1999; Kaletta & Hengartner, 2006). In fact, mutated human presenilin genes lead to the most frequent and aggressive forms of Alzheimer's disease (Wittenburg et al., 2000). C. elegans is used as a model organism to study the genes regulating the developmental process.

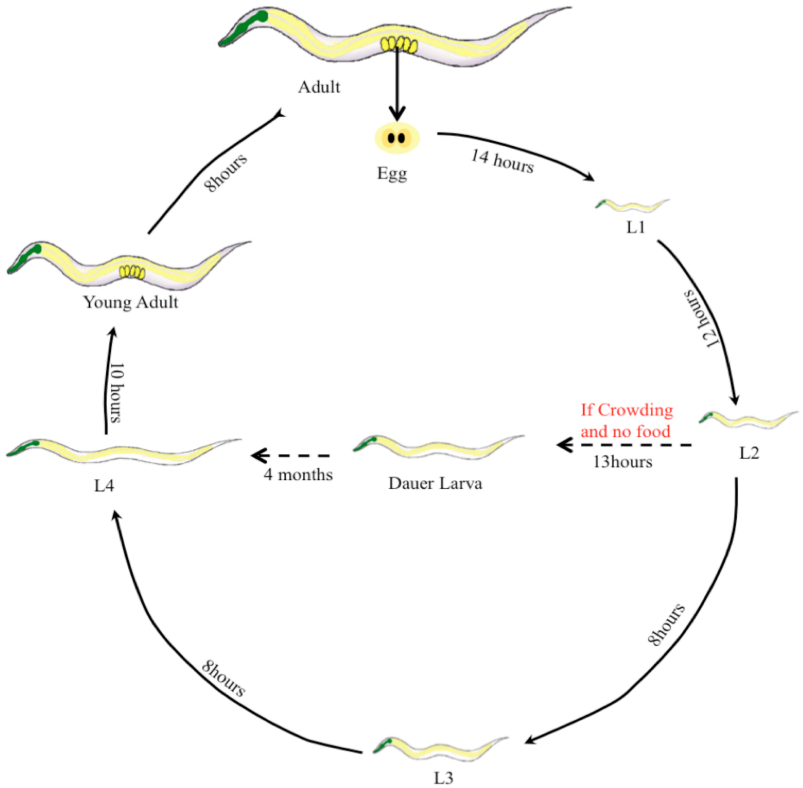

Figure 1: Life cycle of C. elegans at 22ºC (room temperature): Constructed using information from WormAtlas. C. elegans life cycle is temperature-dependent and they usually live about 2-3 weeks at room temperature. C. elegans goes through four larval stages before becoming a

reproductive adult. However, in the absence of food or crowdedness, C. elegans can enter the dauer larval stage, which allows them to survive up until 4 months.

C. elegans has numerous attractive features that make it a powerful model organism for genetic studies. Firstly, it is very easy to cultivate (Kaletta & Hengartner, 2006). Even though in its natural environment it feeds on various bacteria, it can easily be grown and maintained in laboratory conditions with a simple diet of Escherichia coli (Barr, 2003; Brenner, 1974; Kaletta & Hengartner, 2006). We used the OP50 strain of E. coli in our study. Secondly, C. elegans are transparent. They are easy to study under the microscope (Kaletta & Hengartner, 2006; Sulston, Schierenberg, White, & Thomson, 1983). In fact, each cell can be individually observed without needing to cut and fix it to a slide. This property is known as single cell resolution, which means you can look at the animal under a microscope and see every cell within it without having to do anything special or extraneous to the animal. Moreover, the research can clearly trace the entire cell lineage through C. elegans' transparent body (Sulston et al., 1983). Thirdly, C. elegans possesses a fixed number of cells that are invariant, which essentially means that they are nearly identical to each other (Jorgensen & Mango, 2002; Labouesse & Mango, 1999; Sulston et al., 1983). This property is quite advantageous for carrying out experiments because there is very little room for randomness (Sulston et al., 1983). Fourthly, C. elegans has a very short life cycle of about three days at room temperature and it reproduces very rapidly giving birth to more than 300 progeny (Barr, 2003; Kaletta & Hengartner, 2006). Thus, scientists are able to rapidly conduct experiments which otherwise would take time if C. elegans took a longer period to grow and reproduce (Figure 1). Fifthly, C. elegans' small size makes it very easy to handle in the laboratory. It is small enough to be able to be cultivated in the amount of hundreds of worms on a single plate (Kaletta & Hengartner, 2006). It diminutive size also enables it to be easily transferred into a microfuge tube. However, C. elegans are big enough to be individually picked using a standard stereo microscope and platinum wire.

Furthermore, C. elegans have a very interesting gender division. C. elegans have two sexes: hermaphrodites with 959 somatic cells and males with 1031 somatic cells (Gilbert, 2006; Kaletta & Hengartner, 2006; Schedin, Hunter, & Wood, 1991; Sulston et al., 1983). Males are important for genetic studies because they allow us to introduce different alleles into a population by inducing the worms to sexually mate with a worm carrying the desired allele (Figure 2). C. elegans are easy to preserve by freezing desirable strains in liquid nitrogen (Kaletta & Hengartner, 2006). Finally, C. elegans' genome has been completely sequenced (Barr, 2003; Hillier, Coulson, Murray, Bao, & Sulston, J. and R. H. Waterston, 2005; Rose & Kemphues, 1998; Sulston et al., 1983) . Therefore, we have a vast amount of information for apprehending the molecular organization of an organism. Its genome is packed into six chromosomes consisting of about 19,000 genes out of which about 40% have been identified to be homologous to other organisms, including humans (Genome sequence of the nematode C. elegans: A platform for investigating biology.1998; Cooper & Hausman, 2006). Essentially, these characteristics make C. elegans a powerful model organism for developmental genetic studies.

The Importance of Understanding Pharyngeal Development

As mentioned before, C. elegans' pharynx is the foregut, which is an essential part of the digestive tract of the worm composed of muscular epithelial tissue (Albertson & (Mango, 2007) . It is a narrow, tube-shaped organ, which is Thomson, 1976; Horner et al., 1998). Since the publication of ultra-structural studies by Alberston and Thomson (1976), a great amount of information was elucidated about the C. elegans' pharyngeal anatomy and structure (Mango, 2007).

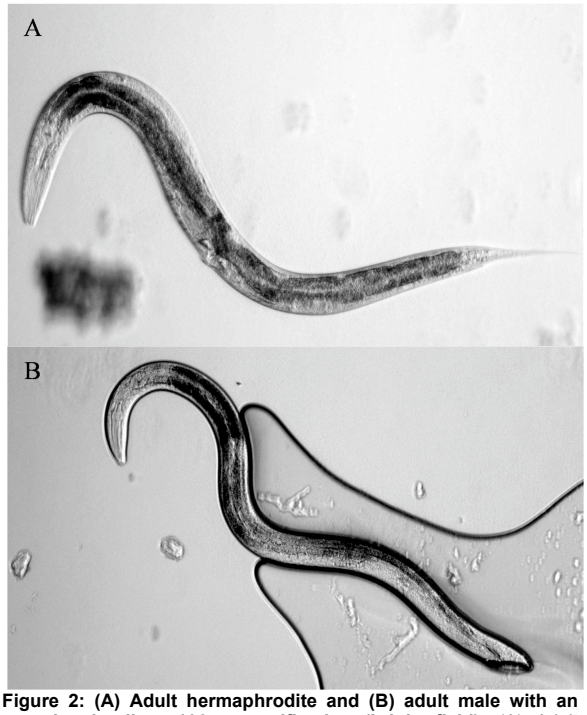

Figure 2: (A) Adult hermaphrodite and (B) adult male with an arrowhead tail at 100x magnification (bright field): (A) Adult hermaphrodite and (B) adult male with an arrowhead tail at 100x magnification (bright field).

The pharynx is a neuromuscular organ that functions as a rhythmic pump to grind and consume bacteria (Horner et al., 1998; Mango, 2007). Food is digested in the pharynx and then, the food is further passed down the gut (Albertson & Thomson, 1976). C. elegans' pharynx has a two-lobed, linear structure. It is divided into different sections from the anterior to the posterior end (Mango, 2007). For example, it is composed of the buccal cavity, procorpus, metacorpus, isthmus and terminal bulb (Figure 2) (Mango, 2007). Moreover, a basement membrane separates the pharynx and demarcates it from other C. elegans' tissues (Albertson & Thomson, 1976). In fact, the basement membrane marks out C. elegans' pharyngeal nervous system as an entirely separate entity that consists of five different types of motor neurons and six different types of inter-neurons (Albertson & Thomson, 1976). C. elegans' pharynx's composition, like the makeup of the foreguts of other complex organisms such as humans, consists of many distinct cell types. It is polyclonal, which means that the pharynx is composed of multiple cell types (Mango, 2007). These cell types include epithelial cells (9), gland cells (5), marginal cells (9), muscle cells (34), and neurons (20) (Albertson & Thomson, 1976).

C. elegans' foregut (pharynx) has become a great tool to study organogenesis that occurs during the developmental stage due to various, multiple appealing features of the pharynx (Mango, 2007). For example, organogenesis can be observed in the pharynx from the beginning to the end (Mango, 2007). Scientists are able to even trace the last steps of differentiation and morphogenesis because C. elegans are transparent and their complete cell lineage is known (Mango, 2007). In fact, the structural anatomy of the pharynx has been well studied and keenly characterized (Albertson & Thomson, 1976). Using certain antibodies and green fluorescent protein (GFP), researchers can mark and track individual pharyngeal cell types and identify various developmental

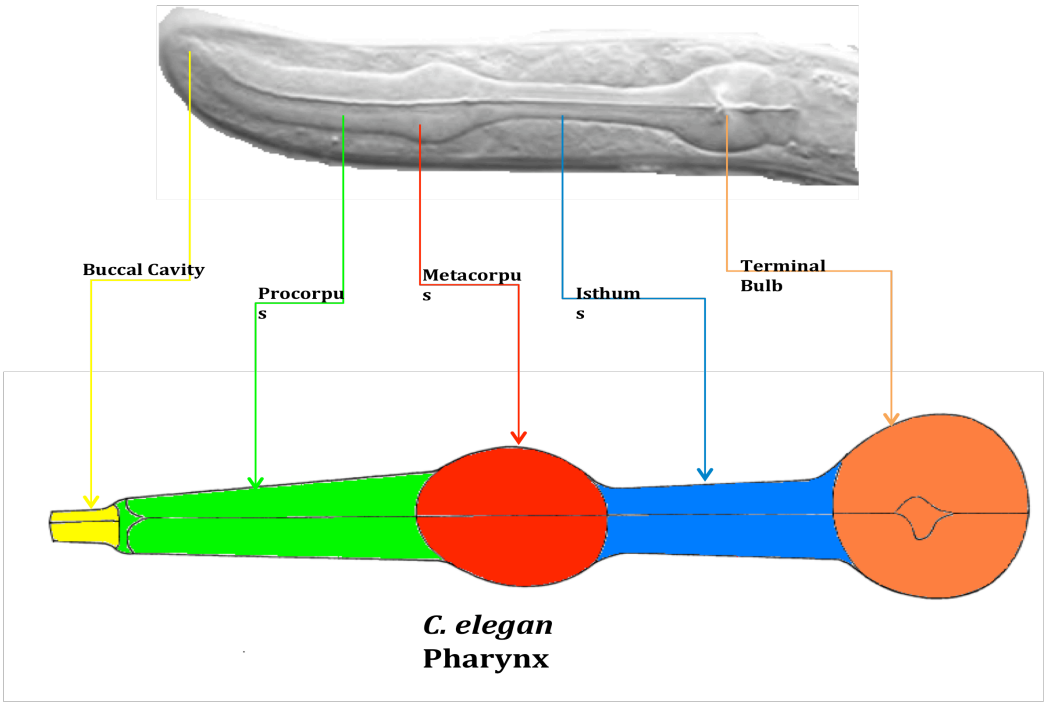

Figure 3: Anatomical sections of C. elegans pharynx: C. elegans pharynx up and its Schematic drawing lower illustrates anatomical sections of pharynx from anterior to posterior: Buccal cavity (lower yellow); Procorpus (lower green); Metacorpus (lower red); Isthmus (lower blue); Terminal bulb (lower orange).

stages the pharynx undergoes during its development (Mango, 2007). In addition, C. elegans' ability to develop a normal, differentiated pharynx even in the face of complications, such as abnormal morphogenesis, in the developmental process allows scientists to focus on components that directly modulate pharyngeal formation (Mango, 2007). Scientists can disregard the possibility of indirectly produced pharyngeal abnormalities by C. elegans' intracellular machinery that regulates other developmental processes. Finally, there are several mechanistic characteristics and evolutionary pathways that C. elegans' pharynx utilizes that are commonly applied by higher organisms, such as humans (Mango, 2007). Therefore, C. elegans' pharyngeal development during organogenesis is ridden by similar developmental obstacles as other higher organisms.

C. elegans' pharynx has shown to have mammalian orthologues or Orthologs, which are genes in different organisms that originate from a single gene in those organisms' last common ancestor. The importance of these genes is that they often maintained identical biological roles and functions in the organisms of modern day (Remm, Storm, & Sonnhammer, 2001). For example, it is thought to be analogous to not only the esophagus and stomach (foregut) of other organisms but it is also found to be analogous to the human heart (Mango, 2007). Both the pharynx and human heart are shaped like a muscular tube that moves essential materials through the body through rhythmic contractions needed to sustain life. There are some key differences between the human heart and C. elegans' pharynx. While the heart pumps blood throughout the body carrying vital nutrients and minerals, the pharynx grinds and ingests bacteria and then, passes the food further down the gut of the worm. However, both organs rely on electrical impulses to maintain synchronous muscle contractions. They do not require any input from the nervous system in order to carry out their function (Haun, Alexander, Stainier, & Okkema, 1998; Mango, 2007). Moreover, they operate using similar types of potassium-and voltage-gated calcium channels (Mango, 2007).

In addition, there have been orthologous transcriptional factors that have been identified in both the human heart and C. elegans pharynx. These transcriptional factors are thought to be essential for the normal development of each of the organ. For example, both the heart and pharynx utilize transcriptional factors belonging to the NKX transcriptional factor family in order to develop (Mango, 2007; Okkema, Ha, Haun, Chen, & Fire, 1997). The NKX transcriptional factor family is a phylogenetically conserved group of homeobox genes, which are approximately 60 amino acid sequences and encode transcriptional regulatory proteins that have vital roles during developmental stages of an organism (Lints, Parsons, Hartley, Lyons, and Harveyand, 1993; Slack, 2006). It has been found that a gene called the ceh-22 gene encodes for an NK-2 class homeodomain protein in C. elegans (Okkema & Fire, 1994). NK-2 class homeodomain protein is required for normal pharyngeal development. Moreover, the ceh-22 gene is functionally similar to Drosophila's tinman and vertebrate's Nkx2.5, which are involved in the cardiac muscle formation in Drosophila and vertebrates (such as zebrafish and humans) respectively (Okkema et al., 1997). Therefore, the ceh-22 gene is responsible for muscle development in C. elegans (Okkema, Ha, Haun, Chen, & Fire, 1997). Moreover, Haun (1998) and his colleagues have successfully shown that worms with a mutation in the ceh-22 gene (the ceh-22 mutants) can be effectively rescued through the expression of the vertebrate's Nkx2.5, which was introduced in the mutant worms, in pharyngeal muscle.

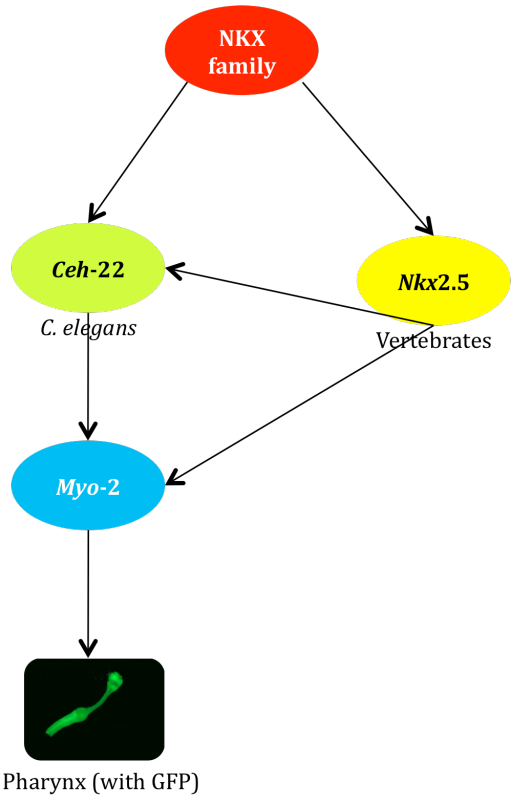

Figure 4: A potential pathway for the Ceh-22 and Nkx2.5 gene: The ceh-22 gene and Nkx2.5 share a similar role in C. elegans and vertebrates respectively. Nkx2.5 can activate the ceh-22 gene and the pharyngeal muscle protein, myo-2, to form pharynx.

The effective rescue of the ceh-22 mutants suggests that the ceh-22 gene and the pharyngeal muscle protein (myo-2) are both activated by the vertebrate's Nkx2.5 (Haun et al., 1998). Refer to Figure 4 for a hypothesized working pathway for the ceh-22 gene and Nkx2.5. Consequently, these findings suggest that the regulating pathways involved in pharyngeal development in C. elegans share common features with the pathway that regulates the cardiac muscle formation in higher organisms, such as zebrafish and humans.

There are other aspects of C. elegans' pharyngeal development that are linked to the development of other organs in other higher organisms. For example, pha-4 gene, whose expression is essential for the pharyngeal development, has been found to be an orthologue to the FORKHEAD gene family and the FoxA gene in Drosophila melanogaster and mammals respectively (Carlsson & Mahlapuu, 2002) . The FORKHEAD gene family and the FoxA gene are crucial for gut development (Gaudet & Mango, 2002; Kalb et al., 2002). Furthermore, another orthologue of the pha-4 gene is the FoxA2 transcriptional factor, which has also been found to play a crucial role in gut formation in all organisms that have been studied up-to-date (Carlsson & Mahlapuu, 2002). In addition, the pha-4 gene also encodes an HNF-3 homolog Ce-fkh-1, which is expressed in all the muscles of the pharyngeal precursor and it has been determined to regulate their fate. Moreover, the HNF-3 homolog ce-fkh-1 has been discovered to be involved in gut development in other organisms (Horner et al., 1998). These results discovered by various researchers collectively illustrate that the transcription factors have been conserved between organisms. Therefore, the underlying genetic mechanistic pathway governing pharyngeal development must be also evolutionarily preserved in C. elegans.

C. elegans pharyngeal development is not only suggested to be similar to the development of the heart in vertebrates but it has been claimed to be similar to kidney tubulogenesis. For example, both the pharynx and kidney undergo apical to basal polarity rearrangement in tubulogenesis (Portereiko & Mango, 2001). These results support the continued efforts to understand pharyngeal development in C. elegans in order to gain insight into the development of complex organs in other higher organisms, such as ourselves.

The Formation of the Pharynx

Aforementioned, the pharynx of C. elegans is formed poly-clonally, which means that multiple progenitor cell types are involved in the formation of the pharynx. Early developmental patterning starts with the sperm fertilizing the egg to form a zygote (the P0 cell) (Rose & Kemphues, 1998). The site of penetration of the oocyte by a sperm becomes the anterior end of the zygote, while the opposite side becomes the posterior end of the zygote (Goldstein & Hird, 1996). The zygote undergoes an asymmetrical division, which results into an anterior blastomere (AB) and a posterior blastomere (P1) (Priess, 2005). While the AB cell divides into ABa and Abp blastomeres, the P1 cell divides into EMS and P2 blastomeres (Gilbert, 2006; Mango, 2007; Priess, 2005). At this stage, there are four types of blastomeres. However, only two of the blastomeres, the ABa and EMS blastomeres, eventually divide to make the pharynx (Mango, 2007). The other two blastomeres, ABp and P2 blastomeres, do not partake in the further development of the pharynx (Mango, 2007). The ABa blastomere produces the anterior pharynx cells whereas the EMS cell produces the posterior pharynx cells. However, the two blastomeres give rise to pharyngeal cells through a completely different molecular pathway. ABa relies on intracellular communication between cells and glp-1 RNA (a Notch receptor orthologue and maternally contributed gene) to produce components that eventually give rise to the anterior pharyngeal cells (Mango, 2007; Priess, 2005). On the other hand, the EMS utilizes the maternally contributed genes skn-1 and pop-1 to produce the components that eventually give rise to the posterior pharyngeal cells (Bowerman, Eaton, & Priess, 1992; Lin, Thompson, & Priess, 1995; Mango, 2007). In addition, it is worth noting that both the ABa and EMS cells also contribute to formation of non-pharyngeal cells, such as the body wall muscle, epidermis, gonad, intestinal cells, and neurons (Sulston et al., 1983).

The Formation of the Anterior Pharynx

C. elegans' pharynx originates from the two descendents of AB and P1, namely ABa and EMS respectively (Mango, 2007). From the point of development of ABa, ABp, P2 and EMS (which henceforth will be referred to as the 4-cell stage), ABa and EMS blastomeres essentially begin to control the development of both pharyngeal cells as well as non-pharyngeal cells, such as epidermis and neurons. At the 4-cell stage, the fate of the divisional remainders of AB, ABa and Abp, are not yet determined. Therefore, ABa and ABp can act like pluripotent stem cells and they can differentiate into any worm body-cell, such as cells that make up the epidermis and body wall muscle (Sulston et al., 1983). However, ABa and ABp, express GLP-1 or LIN-12 receptors, which are Notch receptors orthologues. Notch signaling mechanisms and pathways have been evolutionarily conserved as intact processes during the development of various organisms and Notch signaling has

been observed to play a vital role in determining cell fate as well (Artavanis-Tsakonas, Rand, & Lake, 1999). Likewise in pharyngeal development, Notch signaling pathway determines the anterior pharyngeal cell fate at the 4-cell stage. According to Good et al. (2004), the divisional remainders of AB initially adopt the fate to turn into an ectodermal cell. The posterior daughter of AB, ABp, expresses the glp-1/Notch receptor. It interacts with P2 via Notch signaling pathway. ABp expresses a glp-1/Notch ligand, which is encoded by a maternally inherited gene called apx-1. Through Notch signaling ABp is confined to a fate to become into ectodermal cells (Good et al., 2004; Mango, Thorpe, Martin, Chamberlain, & Bowerman, 1994). The glp-1 targets the ref-1 family of transcriptional factors, which when activated inhibit the activity of a pair of redundant T-box genes, tbx-37 and tbx-38 transcriptional factors. Tbx-37 and tbx-38 transcriptional factors are essential for the pharyngeal development because they activate the pha-4 gene, which determine pharyngeal cell identity (Good et al., 2004). To recap, the glp-1/Notch receptor activation in ABp via Notch signaling pathway activates ref-1 family of transcriptional factors, which in turn suppresses the tbx-37 and tbx-38 activities, which monitor the expression of the pha-4 organ-identity-determining gene.

On the other hand, the anterior daughter of AB, ABa, does not come into contact with P2 and therefore, it does not become confined to an ectodermal cell fate. It gains an ectodermal cell fate during the 12 to15-cell stage. During the 12 to 15-cell stage, the granddaughters of ABa interact with a descendent of EMS, MS, via Notch signaling pathway. The interaction between MS and ABa descendants activates Lag-1, which in turn induces the expression of ref-1 family of transcription factors, which activates the pha-4 gene (Smith & Mango, 2007). The activation of pha-4 gene leads to the formation of anterior pharynx (Smith & Mango, 2007). In this case, the pha-4 gene expression is also induced by tbx-37 and tbx-38 transcriptional factors along with its activation by lag-1. The lag-1 activation induces the expression of ref-1 family of transcriptional factors, which as previously mentioned actually inhibits the expression of tbx-37 and tbx-38 transcriptional factors. The inhibition of tbx-37 and tbx-38 transcriptional factors leads to the deactivation of the pha-4 gene (Smith & Mango, 2007). Therefore, the activation of ref-1 family of transcriptional factors has a negative influence on the activation of the pha-4 gene. However, this is not observed in this scenario oddly enough. The reason for this anomaly is that the activation of various components of this mechanism occurs at different times. The tbx-37 and tbx-38 transcriptional factors are expressed during the 24-cell stage while the expression of ref-1 family of transcriptional factors occurs later during the 26-cell stage (Neves & Priess, 2005). Therefore, ref-1 does not inhibit the expression of tbx-37 and tbx-38 during the 24-cell stage. Hence, the pha-4 gene expression is activated due to the expression of tbx-37 and tbx-38 and activation of lag-1. It is clear that the anterior pharyngeal formation is a complex process that entails intricate time-specific gene interactions and spatially induced Notch signaling.

In addition to playing a crucial role in the anterior pharyngeal cell specification, the MS, produced during the 7-cell stage, is also a mesodermal precursor and it is involved in the posterior pharyngeal cell formation. In general, the MS blastomere is responsible for the development of mesodermal cell types including pharyngeal cells, body muscles and coelomocytes (Broitman-Maduro et al., 2009; Maduro, Broitman-Maduro, Mengarelli, & Rothman, 2007). Hutter and Schnabel's (1994) experiment found that removal of the MS blastomere from the developmental process prior to the second Notch signaling interaction results in the failure to produce pharyngeal cells. Furthermore, inactivation of the MS daughters after the second Notch signaling interaction results in the normal formation of the anterior pharynx but it hinders the development of the posterior pharynx (Good et al., 2004). The removal of the EMS, which gives rise to the MS blastomere, after the first Notch signaling interaction consequently leads to neither the anterior pharyngeal cell formation nor the posterior pharyngeal cell development. These results highlight the importance of the MS blastomere and the vital role it plays in the development of C. elegans pharyngeal muscle cells.

As mentioned previously, the pha-4 gene, which determines the identity of the pharyngeal components, is one of the key genes that regulate *C. elegans* pharyngeal development. The pha-4 gene is activated during the 44-cell stage due to the expression of the tbx-37 and tbx-38 transcriptional factors and the activation of lag-1, which is induced due to the Notch signaling interaction between the ABa descendants and the MS blastomere. When pha-4 gene is activated, it activates different genes at different time intervals (Good et al., 2004). Mango and Lambie, and Kimble (1994) found that inactivation of the pha-4 gene or the loss of function of the pha-4 gene in mutant worms leads to the suppression of C. elegans pharyngeal development. In other words, *C. elegans* pharynx does not form or develop when the pha-4 gene is not activated. However, Arnone and his colleagues found that mutants with an ectopic pha-4 gene expression develop extra pharyngeal cells (Mango, Lambie, & Kimble, 1994). Strangely, embryos with mutant tbx-37 and tbx-38 transcriptional factors have been found to express the pha-4 gene in intestinal and rectal cells, yet many did not demonstrate pharyngeal cell formation (Good et al., 2004). These findings suggest that the pha-4 gene expression is important for the formation of pharynx but it still does not singlehandedly explain how pharyngeal cells, such as muscle cells, are directed to take on their specific fate. Moreover, external factors that govern pharyngeal muscle activity still need to be explained.

The NK-2 family homeobox gene ceh-22 is linked to pharyngeal muscle formation. It is also thought to govern the development of the heart in other higher organisms. The ceh-22 gene, which is activated by the pha-4 gene, is the earliest gene known to be expressed in the pharyngeal muscle cell development and therefore, it is closely associated with other genes that play a role in specifying pharyngeal muscle cell fate (Vilimas, Abraham, & Okkema, 2004). The ceh-22 gene activates the myo-2 gene, which is responsible for producing pharyngeal-muscle-specific myosin protein (Okkema et al., 1997; Vilimas et al., 2004). Okkema et al. (1997) experimentally found that the ceh-22 gene function loss leads to a weak, thinner, and less distinct pharynx than observed in wild type nematodes. The malformed pharynx resembles the pharyngeal phenotype of worms with a defective feeding pharyngeal phenotype because of the abnormal pharyngeal musculature (Okkema et al., 1997). However, pharyngeal muscle cells are still present in mutants with a ceh-22 gene mutation, which suggests that there might be other factors contributing to the anterior pharyngeal muscle formation. Smith and Mango (2007) found that tbx-2 is another major player in the anterior pharyngeal muscle fate specification. Tbx-2 shows a similar phenotypic result as the phenotype of the ceh-22 mutant worms. In fact, the worms with an inhibited tbx-2 or a loss of function of tbx-2 have little or no anterior pharyngeal muscle cells but these worms still possess an intact posterior pharynx (Smith & Mango, 2007). Smith and Mango's research findings suggest that proper coordination of the tbx-2 and pha-4 gene is essential for the successful specification of pharyngeal muscle fate. However, research results of Vilimas et al. (2004) suggest that an enhancer sequence

plus pha-4 gene is crucial for the specification of pharyngeal muscle cell fate. It appears that the pha-4 gene is a vital gene for the determination of pharyngeal muscle fate and it works synchronously with other genes, such as the tbx-2. There might be a chance that the pha-4 gene might utilize more than one pathway for anterior pharyngeal muscle formation.

The Formation of the Posterior of Pharynx

The development of the posterior of the pharynx does not depend on the intercellular interactions of glp-1/Notch signaling like that of the anterior pharynx; it rather depends on the mesodermal precursor MS cells that utilize a Notch-independent pathway (Bowerman et al., 1992; Maduro, Kasmir, Zhu, & Rothman, 2005). At the 4-8 cell stage, the endomesodermal precursor EMS lineage receives signals from two maternal genes, skn-1 and pop-1, to develop pharyngeal cells. At this stage the EMS blastomere cells are specified by bZIP/homeodomain transcription factor skn-1 that encodes bZIP-related transcriptional factors. More specifically, skn-1 specifies the two daughters of EMS blastomere, the anterior daughter MS, which primarily form mesodermal cells and the posterior daughter E, which is endodermal precursor and forms the entire intestine (Broitman-Maduro et al., 2009; Maduro et al., 2005), through EMS blastomere by activating med-1 and med-2 transcriptional factors. Med-1 and med-2 transcriptional factors are essential for activation of mesodermal identity genes that specify MS blastomere. Moreover, the activation of med-1 and med-2 also activates a new T-box transcriptional factor called tbx-35, which in turn is thought to be involved in the activation of organ identity gene pha-4 in MS blastomere (Bowerman et al., 1992) (Figure 5). This is due to tbx-35 mutant worms failing to develop the posterior pharynx. A recent experiment (Broitman-Maduro et al., 2009) revealed that a NK-2 class homeobox gene ceh-51 is a direct target of TBX-35, where tbx-35 activates ceh-51, and both then activate genes of interest in MS blastomere development, because the removal of tbx-35 and ceh-51 together leads to similar results as does the removal of med-1 and med-2, where MS derived tissues are greatly decreased. Also, ablation of skn-1 results in EMS descendents adopting a cousin of EMS blastomere fate, the C blastomere, as well as resulting in the total absence of pharynx because C blastomere does not produce Notch ligands to induce ABa (to form posterior pharyngeal cells) as well as the EMS (to form the posterior pharyngeal cells) (Lin, K. T., Broitman-Maduro, G., Hung, W. W., Cervantes, S., & Maduro, M. F., 2009). The C blastomere intern leads to muscle tissue, hypodermis, and neurons, yet not any posterior pharyngeal cells. Since the MS blastomere is also involved in the signaling of the anterior pharyngeal cells

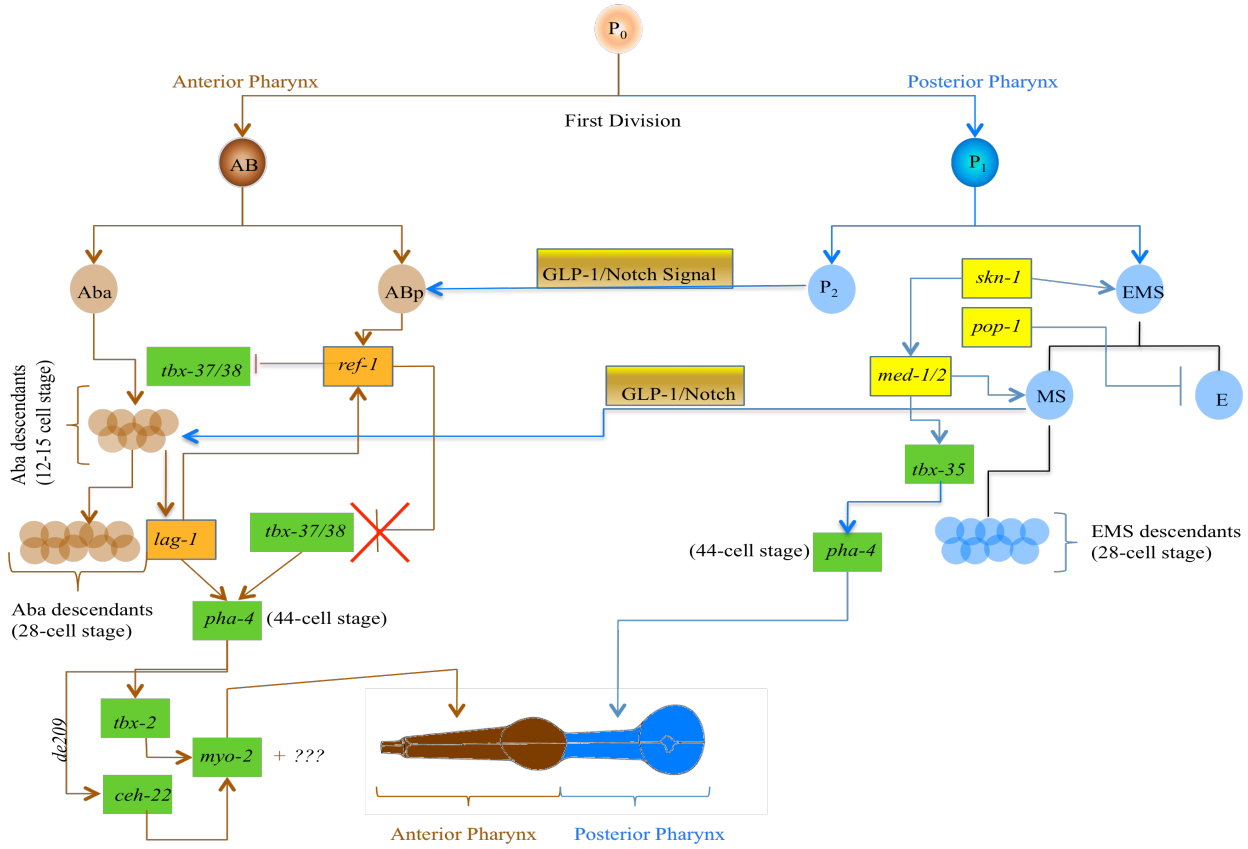

Figure 5: Pharyngeal cell signaling pathways in its development: Genes currently known to be involved in AB descendents (brown) and P1 descendents (blue) descendents to activate organ identity gene pha-4, which leads to the formation of the anterior and posterior pharyngeal cell fate. Lines indicate cell divisions. Blue color indicates association to the posterior pharynx and brown color the association to the anterior color. Genes colored orange are identified to be more specific to the anterior pharyngeal development. Genes colored yellow are identified to the more posterior pharyngeal cell, while genes colored in green have been identified to have a role in both anterior and posterior pharyngeal cell development. This was adapted from (Charron, 2010; Ferrier, 2009) and also utilizes the findings of (Bowerman et al., 1992; Broitman-Maduro et al., 2006; Broitman-Maduro et al., 2009; Good et al., 2004; Labouesse & Mango, 1999; Lin et al., 1995; Maduro et al., 2005; Maduro et al., 2007; Maduro, 2009; Mango, 2007; Neves & Priess, 2005; Priess, 2005; Smith & Mango, 2007; Lin, K. T., Broitman-Maduro, G., Hung, W. W., Cervantes, S., & Maduro, M. F., 2009)

specification, it also hinders the formation of the anterior pharyngeal cells.

Similarly to skn-1, pop-1 plays an essential role in the EMS blastomere specification. Pop-1 specifies the anterior sister cell fate, because during the development, when cells are divided and differentiated along the anterior-posterior axis, it is observed that pop-1 is being more commonly expressed in the anterior sister cells (Labouesse & Mango, 1999). Interestingly, EMS is one such cell that divides anterior-posterior, where the anterior cell is the MS that expresses pop-1. The expression of pop-1 at this point here then inhibits genes responsible for the E blastomere fate (Broitman-Maduro, Lin, Hung, & Maduro, 2006). However, pop-1 loss of function, results in MS blastomere mis-specification and leads to E-like blastomere cell fate, because pop-1 usually inhibits the activity of endoderm promoting genes end-1 and end-3 in MS blastomere that are responsible for the endoderm development (Broitman-Maduro et al., 2009; Lin, K. T., Broitman-Maduro, G., Hung, W. W., Cervantes, S., & Maduro, M. F., 2009). As a result, when pop-1 is inactive, the formation of endoderm tissue fate is promoted instead of posterior pharyngeal cell fate (Lin et al., 1995; Maduro et al., 2005). However, the repression of endodermal fate by pop-1 in E blastomere is overcome by Wnt/MAPK signaling that results in phosphorylation and export of pop-1 from the E nucleus (Maduro et al., 2005). In short, posterior pharynx formation depends on med-1 and med-2 transcriptional factors (which are activated by skn-1) inducing the MS blastomere fate through activation of tbx-35, that in turn activates pha-4 to develop the posterior pharynx.

Differentiation and Cell Fate: How a single cell develops into a multicellular organism?

A fundamental question in developmental biology is how can a single cell give rise to countless specialized cells with particular functions that eventually make up an individual. Upon completion of fertilization, which is marked by the fusion of a sperm into an egg, the development of multicellular organisms proceeds through a series of mitotic divisions called cleavage (Gilbert, 2006). During cleavage, the egg cytoplasm is continuously divided into many smaller and nucleated cells called blastomere, whereby the cytoplasmic volume remains unchanged (Gilbert, 2006). As a consequence, the cytoplasmic components are distributed unevenly into blastomeres. Moreover, these unevenly distributed cytoplasmic components are often transcriptional factors that control the activation or repression of specific genes in the blastomeres that they are acquired. As a result, distinct groups of specific cells with specific developmental goals or programs arise when different nuclei in a different blastomere are exposed to these factors (Gilbert, 2006). These determined groups of cells eventually become specialized parts of an animal, like the *C. elegans* pharynx, and together make up an entire organism.

In general, the cell fate of an organism is determined by several factors, such as contribution of maternal RNA, zygotic gene regulation, intercellular signaling, and the position of the cell in relation to its neighboring cells (also known as conditional specification) (Gilbert, 2006). The process in multicellular organisms, like *C. elegans*, begins with the zygote utilizing maternal RNA to provide initial instructions for the specialization and differentiation of subsequent cells, such as AB and P1. The AB and P1 then use zygotic gene regulation, otherwise known as differential gene expression, to continuously restrict cell fate during embryogenesis (Gilbert, 2006). It is worth noting that the *C. elegans* pharynx development exhibits both autonomous (P1 blastomere dependent on Skn-1 gene) and conditional (AB blastomere dependent on Glp-1 signaling) specification (Gilbert, 2006). This was observed when the two blastomeres were experimentally separated, where P1 developed normally all the posterior pharyngeal cells without the presence of the AB blastomere, whereas the AB blastomere only developed a fraction of the anterior pharyngeal cells (Priess & Thomson, 1987). As cell division is continuous, the intercellular signaling, as well as the position of cells, is crucial for genetic regulators to fulfill their role in determining cell fate. Together, maternal RNA, zygotic gene expression, intercellular signaling, and the placement of the cell during development influence each cell's fate (Gilbert, 2006).

Morphogenesis

The development of a multicellular organism is indeed a complex process, which depends on various components and pathways to come together and work properly. One of these processes, which is vital for the development of an organism, is called morphogenesis. Morphogenesis is a process that is marked by cellular movement and ensures the proper differentiation and growth of specialized tissues and organs (Gilbert, 2006; Seydoux & Greenwald, 1989). The precise regulation of cell migration and shape are essential for the formation of the three-dimensional structures of tissues and organs (Portereiko & Mango, 2001). The *C. elegans* pharynx morphogenesis is initiated by the end of gastrulating when cell division is nearly complete. At this stage, the first recognizable pharyngeal cells, primordium cells that are attached to each other by adherence junctions, are visible as a ball of cells attached to the midgut cells in the C. elegans embryo also by the adherence junctions. The pharyngeal primordium, however, is not attached to the buccal cavity (oral cavity) at this point. Thus, similarly to tubulogenesis of heart, kidneys, and digestive tract, these cells shift in position and orientation to form a linear tube that connects the digestive tract to the exterior of the embryo, and therefore, forming a linear tube that is connected anteriorly by epithelium to the buccal cavity and posteriorly to the midgut (Portereiko & Mango, 2001).

The *C. elegans* pharyngeal morphogenesis is marked by a ball of primordium cells, which have to elongate and develop into a narrow tube of pharyngeal cells that are attached to each other by adherens junctions (Albertson & Thomson, 1976). Pharyngeal morphogenesis can be divided into three distinct stages. The first stage is called reorientation stage, in which most anterior pharyngeal epithelial cells rotate and rearrange their position and alter their polarity. This leads to alteration of pharyngeal morphology from cyst to a short-linear tube and the alignment of the pharyngeal epithelial cells with the arcade cells (Portereiko & Mango, 2001). The second stage of pharyngeal morphogenesis is coined as epithilization. In this stage, the epidermis and digestive tract develop a continuous epithelium due to the formation of the buccal cavity adherens junctions that connect the buccal cavity to the pharynx and epidermis (Portereiko & Mango, 2001). Finally, the third stage of pharyngeal morphogenesis is known as the contraction stage. In this final stage, the pharynx, buccal cavity, and epidermis undergo a contraction that brings them closer in proximity. This is as a result of movement of the pharynx anteriorly and the epidermis of the mouth posteriorly (Portereiko & Mango, 2001). In sum, during pharyngeal mophogenesis, a ball of cells undergoes a thorough reorientation, epithilization, and contraction in order to become a functional bi-lobed organ that we know as the pharynx.

Morphogenesis goes hand-in-hand with cell adhesion. Cell adhesion molecules are essential for the cells to adhere to each other during morphogenesis, because they provide cells with a stable environment and allow them to migrate and thereby form three dimensional

structures, like the *C. elegans* pharynx (Cox & Hardin, 2004; Gilbert, 2006; Gumbiner, 1996). Also, cell adhesion molecules, such as integrins, a family of transmembrane receptors that promote cell-cell and cell-matrix adhesion, play a crucial role in promoting morphogenesis by causing neighboring cells to initiate cell migration (Hillis & Flapan, 1998; Huveneers et al., 2007; Huveneers, Truong, & Danen, 2007). In addition to cell migration, integrins also play an essential role in cell fate, differentiation, proliferation, and programmed cell death (apoptosis). The improper morphogenesis along with improper functioning cell adhesion complexes, such as integrins, can lead to a number of diseases, like cancer, rheumatoid arthritis (inflammatory disorders), inflammatory bowel disease, and asthma, as well as thrombosis (cardiovascular disease) (Huveneers et al., 2007).

There are many factors that are observed to alter *C. elegans* pharynx morphogenesis. It has been observed that the loss of arcade cells leads to the failure of the contraction stage of the morphogenesis. Furthermore, there are many genes seen to affect pharynx morphogenesis when their functions are experimentally altered. For example, an ETS-domain transcriptional factor homologue, ast-1, loss of function results in pharynx unattached embryo with inability to feed (Mango, 2007; Schmid, Schwarz, & Hutter, 2006). Depletion of another gene called pha-2, leads to abnormally thick pharynx (Mango, 2007; Mörck, Rauthan, Wågberg, & Pilon, 2004). The eya-1 gene mutation leads to the worms' death at L1 or L2 stage with thin pharynx phenotypes and feeding defects (Daniels, 2007; Furuya, Qadota, Chisholm, & Sugimoto, 2005; Mango, 2007). Finally but not limited to, sma-1 mutants (a gene that is homologous to BH-spectrin and important for the elongation of pharynx) are viable but morphologically exhibit short pharynx and short bodies (Mango, 2007; McKeown, Praitis, & Austin, 1998). As can be seen, morphogenesis of pharyngeal cells can be affected by many factors and understanding of pharyngeal morphogenesis in *C. elegans,* can lead us to a better understanding of morphogenesis in higher organisms such as humans.

Organogenesis

Organogenesis refers to the time during embryogenesis when the organs are being developed (Gilbert, 2006). Organs are vital structures composed of many different cell types and tissues that are spatially and functionally organized into a unit to sustain a life. Therefore, it is vital for this process to take place correctly because malformed organs can lead to an organism's death. Formation of an organ, such as the C. elegans pharynx, is a complex process, which involves activation and deactivation of many genes. For a group of cells to develop into a functional organ, they have to undergo proper cell-to-cell communication to differentiate and acquire a proper fate, followed by and assembly into tissue through appropriate morphogenesis (Gilbert, 2006; Mango, 2007). In sum, strictly controlled processes, such as differentiation and cell commitment, intercellular signaling, as well as morphogenesis are required for a functional organ, like the C. elegans pharynx, to develop.

Gap in knowledge

Although a great amount of information has been learned about the *C. elegans* pharynx development, a wealth of knowledge still remains to be uncovered about the functions and mechanisms of genes that are involved in cell fate specification and morphogenesis of pharyngeal muscle cell development. Therefore, the goal of this research was to understand the genetic and molecular mechanisms involved in pharyngeal muscle development.

Our lab had previously induced 265 point mutation in C. elegans pharynx using ethylmethanesulfonate (EMS), which is a technique that introduces point mutation in DNA, to study and identify genes that are vital to the pharynx as a whole and in particular to the posterior pharyngeal cell development. Furthermore, our lab has previously completed a genetic screen for worms with abnormal pharynx muscle morphology induced my EMS. The worms have an integrated myosin heavy chain structural gene with green fluorescent protein (myo-2::GFP) reporter gene, which enables rapid identification of worms with misshapen or missing pharynx. We have screened about 10,000 haploid genomes and identified close to 200 mutant lines. We are now focusing on two major classes of mutants: ones with a short, wide pharynx and others with amorphously shaped pharynx muscle cells.

Twenty of these mutants manifested short and wide blunt pharynges (Ferrier, 2008). These worms most likely have mutated genes that are responsible for embryonic elongation of the pharynx. We have mapped and located many of these mutations to certain small regions of a particular chromosome through a single nucleotide polymorphism mapping (SNP mapping). More specifically, our lab had previously established linkages for 10 of these mutants, of which 4 of them turned out to be homozygous recessive mutants exhibiting a blunt head phenotype. One of these blunt head phenotypes is exhibited by a strain called mutant 77 (M77), which was chosen to be the subject of this study. We have identified two mutant lines as allelic forms of sma-1, a beta-spectrin. Many other mutant lines are not located on any of the previously described short-pharynx gene loci. Moreover, most of these mutant lines are larval lethal. In fact, acrylic bead feeding assays have shown that they are unable to ingest food. Further mapping is being conducted to identify the actual genes responsible for the phenotypes.

Currently, the subject of this thesis, the mutation resulting in the mutant phenotype observed in M77, is genetically traced. The M77 worms exhibit a mutated short and wide pharynx with differentiated pharynx muscle cells. We hypothesize that through the use of complementation analysis and genetic mapping we will identify the location of the gene, and through antibody staining, we will reveal the structure of the pharynx and determine the identity of the gene causing the M77 mutation. We further hypothesize that through complementation analysis, we will genetically balance the M77 allele.

Methods and Materials

C. elegans Media Protocol

Nematode growth medium (NGM) plates were made as follows: for every one liter of the medium, 3 grams of NaCl, 17 grams of Bactoagar, 2.5 grams of Bactopeptone, and 1 milliliter of cholesterol (5mg/mL in EtOH) were added into a 5 liter flask and dissolved in 975 ml of distilled water. Next, a stirring bar was placed into the flask and the mixture was autoclaved for 60 minutes. Afterwards, the flask containing the mixture was mixed thoroughly and cooled down sufficiently to touch the exterior of the flask by hand comfortably without burning (usually 15-25 minutes). Using sterile technique, the following substances were added: 1 ml of 1 M CaCl2 1 ml of 1 M MgSO4, and 25 ML of 1 M potassium phosphate buffer (pH 6.0). Finally, the medium was added in 11 ml increments into 60 mm Petri Dishes or plates (60 mm) and in 4 ml volume into 35 mm Petri Dish (mating plates) (35 mm). The plates were then allowed to solidify for a few hours and 250 µL of OP50 Escherichia coli (E. coli) were added and spread into the large plates, and 10 µL of OP50 E. coli into the mating plates as a dot.

Growth and Culturing of C. elegans

BC4637 (genotype: sDf130(s2427) unc-32(e189) III; sDp3 (III;f)), BC4697 (genotype: sDf121(s2098) unc-32(e189) III; sDp3 (III;f)), Hawaiian CB4856 (genotype: C. elegans wild type, CB subclone of HA-8 (Tc1 pattern IX)), CB4681 (genotype: nDf17/qC1 dpy-19(e1259) glp-1(q339) III), MT690 (genotype: nDf6/unc-93(e1500) dpy-17(e164)III), MT696 (genotype: nDf12/unc-93(e1500) dpy-17(e164)III), MT699 (genotype: nDf15/unc-93(e1500) dpy-17(e164)III5), NG2618 (genotype: yDf10 unc-32(e189)/qC1 dpy-19(e1259) glp-1(q339) III), TY1353 (genotype: yDf10 unc-32(e189)/unc-93(e1500) dpy-17(e164)III), and PD4792 (genotype: mIs11 IV) strains were used in this research project (Wormbase WS210, 2010). All C. elegans strains, except the M77 strain, were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, Minneapolis. Mutant strain M77 and some of the deletion strains, such as BC 4637, M696, M690, and NG2618 (which were later reordered from CGC), were obtained from the previously frozen stock at Lake Forest College. The strains were maintained via worm transfer into new 6 cm NG plates with 250 µL of E. coli OP50 plates at various temperatures of 10˚C, 12˚C, 14˚C and 20˚C—25˚C (room temperature) in order to control a worm's growth period.

The worm transfer was done as follows: E. coli OP50 was added to the edge of a worm pick (a 1-3 cm piece of platinum wire attached to either a pasture pipette or a premade worm pick with the edge bent horizontally to the pasture pipette) and then used to gently touch a worm, which would very easily stick to it. Once the worm was on the pick, it was then transferred to a new plate by again gently touching the edge of the pick to the area of the plate with food (E. coli), and held a few seconds for the worm to crawl off of the pick. Mutant and deletion strains were maintained by picking 3-5 worms with particular phenotypes, such as wild-type hermaphrodite (for the deletion strains phenotypes described in worm base while for the M77 strain wild-type (WT) heterozygous worms) from a known worm plate, into new plates about every two weeks under a LEICA MZ16 stereo microscope. The plates were then screened for the desirable phenotypes of progenies. For example, in the case of M77, the plates were screened for mutants (with rounded, short dumpy phenotype) and then marked on the plates as mutants. In addition, Hawaiian males were maintained by picking 5 male worms and 3-5 hermaphrodite worms into either 35 mm or 60 mm plates.

Maintaining and Obtaining Heterozygous M77 male worms

M77 male worms were essential for this research project because they were the means of introducing our mutant allele into deletion strains by mating. Males are usually produced by the gametes that lack one of the two X chromosomes (Nullo-X—XO) observed in normal hermaphrodite worms. The XO gametes are usually generated during meiosis as a result of spontaneous chromosomal non-disjunction of the X chromosome, which occurs very rarely in hermaphrodite development. Therefore, due to the predominantly hermaphrodite germ line in C. elegans, males are seen very infrequently in the hermaphrodite cultures, which reproduce by hermaphrodite self-fertilization (Lints and Hall, 2009).

However, there are several laboratory techniques to obtain male worms by causing spontaneous non-disjunction of the X chromosome in hermaphrodite worms. One of these methods, which our lab has been using, is picking 10 L4 stage M77 or PD4692 worms into 60 mm plates and incubating them for 6 hours at 30˚C (Figure 6 (B)). After the incubation period, the plates were maintained in room temperature and the F1 generation was screened for male worms. Another technique, which proved to be more efficient, was treating 10-15 L4 stage M77 worms with 7% ethanol in M9 buffer for 30 minutes, which was then followed by exactly the same procedure as for heat shocking (Figure 6 (A)) (Lyons and Hecht, 1997).

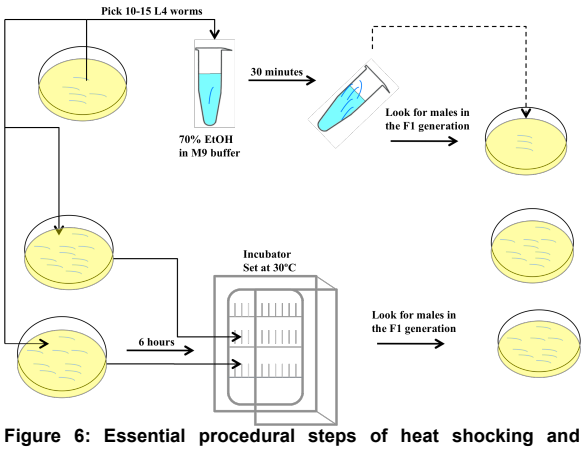

Figure 6: Essential procedural steps of heat shocking and ethanol treatment procedures to induce *C. elegans* male worms. Schematic drawing of necessary procedural steps of (A) 7% ethanol treatment and (B) heat shocking in order to obtain *C. elegans* male worms

SNP mapping (Single Nucleotide Polymorphism mapping)

SNP mapping was carried according to Davis et al. (2005). SNP mapping usually is done in two phases, the chromosome mapping, which narrows down the physical location of a mutant gene to a particular chromosome (in the case of C. elegans in one of the six chromosomes) and interval mapping, which further narrows down the known physical location of a desired mutation gene to a very small mapping unit area (Davis et al., 2005). The physical location of M77 allele has already been determined by my colleague Andrew Ferrier to be in chromosome III. Therefore in this research project, the interval mapping was carried out.

Interval Mapping

We crossed M77 hermaphrodite worms (derived from Bristol N2) with Hawaiian male worms (usually 10 to 10) on a mating plate (Figure 7). After 24 hours, we transferred each hermaphrodite exhibiting a copulatory plug, which ensures the success of mating, into a new 60 mm plate. The first generation (F1) was then screened for male worms after 3 days to further ensure the success of mating. Next, on the sixth day, 96 F2 generation mutant worms were picked into 96 individual Polymerase Chain Reaction tubes, each containing 5 µL of proteinase K (6 mg/ml) diluted 1:10 with worm lysis buffer. Subsequently, the PCR tubes containing the worms were placed in -80˚C freezer for at least 10 minutes (but could be left there for days) before proceeding to the next stage. Next, the PCR tubes were removed from the freezer and placed into the PCR machine (S/N/Part No. 533140319) to be incubated at 65˚C for an hour followed by 15 minutes at 95˚C to inactivate proteinase K. In the mean time, in addition to 2 µL of 100 µM primer set (2 µL forward primer and 2 µL reverse primer) for a specific chromosomal region, the following substances were added into a separate new 1.5 mL PCR tube: 400 µL of 2X Taq Mastermix, 560 µL of nanopure PCR qualified water, and 40 µL of MgCl2. The mixture was then thoroughly mixed and 10 µL of this mixture added into each 96 well of a PCR Thermowell 96 Well Plate (Part number: 6551 and Lot number 18104023) followed by the placement of the caps and centrifugation at 1500RPM.

When the thermocycling was completed, 0.5 µL of the mutant DNA were added to each individual well containing 10µL of the master mix mixture (and the rest stored at -80˚C for later use) followed by another centrifugation at the same speed. This was to ensure that the DNA and the mixture were in the bottom of the plate. Next, the PCR plate was placed into the thermocycler machine to undergo PCR. The thermocycler machine was used to carry out the following three steps. First, Initialization step for 2 minutes at 94°C and 37 cycles of 15 seconds at 94°C to break the hydrogen bonds that hold the DNA together in order to create single stranded DNA. Next, the Annealing step for 45 seconds at 60°C, allowing the primers base-pair with template strands. Finally, the Elongation step for 1 minute at 72°C followed by another 5 minutes at 72°C, where Taq polymerase extends strands of DNA that are complimentary to each of the template DNA strands in the 5 to 3 direction while any mismatched primers will not be extended and consequently dissociated from the template strands. This entire process took 2 hours and then 6 µL of DNA digest mixture was obtained by mixing 420 µL nanopure H20, 160 µL NEB4 buffer, 10 µL BSA and 35 µL DraI restriction enzyme was added to each well followed by incubation at 37°C using thermocycler cycler protocol (37°CDEG6HR) for 6 hours.

The DNA was then separated by electrophoresis (a technique that allows separation of DNA by size or by charge) and stained to visualize and analyze the products by adding 5 µL of orange into the each 96 Well Plate after the digestion step was completed. Subsequently, this mixture was centrifuged at about 1500 rpm to ensure a proper mixture of dye with the rest of the mixture. Finally, 10 µL of sample from each well were loaded, skipping the first well, onto 2.5% agarose gel, which was composed of 5 grams of agarose dissolved into 200 mL of TAE (Tris-acetate-EDTA) or TBE (Tris-Borate-Edta) buffer, microwaved (in Montgomery Word Serial No. 130453) for about 2-3 minutes (or till it boiled) until the agarose was dissolved in the buffer and the buffer (liquid) appeared clear. Next, 20 µL of 10 mg/mLethidium bromide (C21H20BrN3) were added into the dissolved agarose, mixed well, and cooled down to about 65°C before pouring it into the gel tray. Finally, 100 bp ladder was loaded into the first well of each row, and the gel was run at 125 V for 75 minutes. The picture of the gel was then taken using VersaDoc Imaging System (Model No. Versa Doc Imaging System 300TM and Serial No. 590BR0046) and analyzed using known band patterns for Hawaiian and Bristol DNA to determine the recombination [1]frequency, which is the frequency at which crossing over of two alleles for a particular gene occurs. The recombination frequency can be determined by dividing the number of wells illustrating recombination by the total number of chromosomes present.

Complementation analysis

The Complementation test between M77 and deletion worm strains (MT690, MT696, MT699, NG2618, TY1353, CB4681, BC4637, BC4697) was carried by first either crossing 5 to 10 PD4792 male worms or 5 to 10 M77 male worms with 10 heterozygous M77 hermaphrodites in order to obtain a desirable heterozygous M77 male worms. Deletion worm strains refer to a type of mutation that involves the loss of genetic material from a region of a particular chromosome, in this case chromosome III. After 24 hours at room temperature , hermaphrodites were transferred into new individual plates. The F1 generation was then screened for mutants after 2.5-3 days to ensure that the hermaphrodite

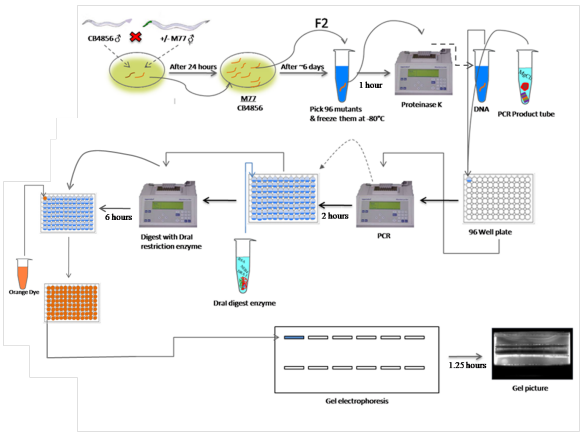

Figure 7: SNP mapping procedure. Schematic drawing of interval mapping necessary protocol steps. From top left to bottom right, two strains of *C. elegans* differing in known single nucleotide polymorphisms are mated and the resulting progeny are processed using polymerase chain reaction; their DNA is then analyzed by *DraI* digestion and agarose gel examination.

worm was indeed heterozygous; and that male worms were present to confirm that mating had taken place. Each phenotypically WT male worm (with 50% probability of being heterozygous for M77 mutation) was then crossed with 5 hermaphrodite deletion strain worms (e.g., 5 NG2618 worms) that carry a particular deletion. The worms were allowed to mate for 24 hours and then each hermaphrodite was transferred into a new 60 mm plate. The F1 generation of these transferred deletion hermaphrodites were then screened after 2.5 or 3 days to establish either success of complementation or failure to complement (Figure 8).

Immunocytochemistry: KT-14, KT-16, KT-20, KT-36, MH4, and MH27 Antibodies

We used several procedures to pre-clean microscope glass slides, such as washing slides with acid, polylycine, even gelatin plus chromium mixture, in order to make slides sticky. In general a mutant plate with a large number of M77 embryos and L1s was washed with M9 buffer three to five times to remove live worms. The first washes were placed into a centrifuge tube, containing L1s. The subsequent wash was made to remove all the big worms, including the L1s to get only the embryos. After all the big worms were washed off, the remaining embryos were then removed by adding 1.0 mL of M9 buffer, rubbing gently with a finger and removing the M9 buffer to a 1.5 mL microfuge tube. In both cases, in the case of L1s or in case of embryos, the tube containing worms was centrifuged at 1000-1500 rpm for 30 seconds. The M9 buffer floating on the top of the centrifuge tube was carefully removed, without removing the worms from the bottom of the centrifuge tube. Additional M9 buffer was added into the centrifuge tube, and centrifuged again, followed by the removal of M9. This procedure was repeated several times until all the worms in the bottom of the tube and the M9 buffer appeared clear.

After the last wash, another 1 ml of M9 buffer was added and 50 µL of this M9 buffer containing either L1s or embryos were removed to a glass microscope slide and 2% pFA (paraformaldehyde) was added into the area of the slide containing worms and the slides were covered with coverslips. Subsequently, the excess pFA was removed from the slides using regular pipet. The slides were then placed in the humidity chamber for 20 minutes. Next, the slides were frozen in liquid nitrogen (at -80°C) for 10 minutes. The coverslips were removed and the slides were

[1] All the experiments were carried out at room temperature (20˚C—25˚C) unless otherwise stated.

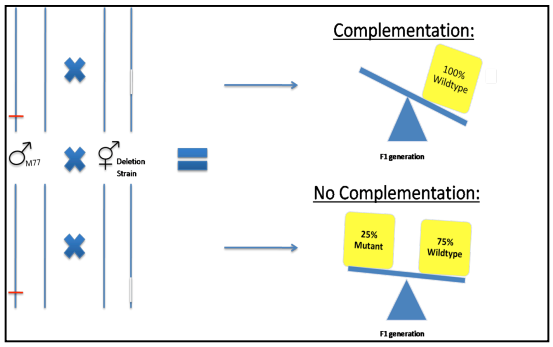

Figure 8: Schematic drawing of complementation test. When the M77 worms are crossed with a deletion strain, then the M77 gene either will complement or does not complement the deletion genes. If the deletion does not overlap with the defective M77 gene, no mutant worms would be observed in the F1 generation and this phenomenon is known as complementation. However, if the M77 defective gene is located in the same chromosomal region as the deletion, then the two overlap, and mutant worms would be seen in the F1 generation that is known as no-complementation.

placed into pre-chilled 100% methanol on ice for 3 minutes. After 3 minutes, the slides were pre-incubated with TNB/10% plus NGS (normal goat serum) in the humidity chamber for one hour. Next, each chosen primary antibodies were diluted in to solutions of TNB/10% plus NGS at a 1:3 dilution. Eachantibody was also used at a 1:100 dilution, as well as with the GFP at a 1:200 dilution. Next, excess TNB/10% NGS was removed from slides and primary antibodies were added into each slide and placed in the humidity chamber and left overnight. The next day, each slide was washed three times in TBS buffer (made using 150 mM NaCl and 10 mM Tris with the pH 8.0) for five minutes. Upon completion of the washes, excess TBS was removed carefully from the slide from all the regions of the slides by using Kimwipes, except where the worms were located. Thereafter, secondary antibodies, one for GFP one for selected antibodies, were diluted to 1:200 with TNB/10% NGS, and 200 µL from it was then added to each slide. Again, they were placed in the humidity chamber for an hour, followed by three time washes in TBS for five minutes, and the removal of excess TBS by Kimwipes. Finally, a drop of mounting media was added to each slide, the slides were covered with coverslipes, and sealed using nail polish. Lastly, the slides were observed under a Nikon Eclipse TE2000-U microscope and the picture of stained worms was taken.

Balancing the M77 allele

To balance the M77 strain, we carried out complementation analyses test. The procedure was followed as described above until observation of F1 generation of crossed between deletion hermaphrodites and heterozygous M77 male worms (Figure 9). In sum, we crossed M77 heterozygous male worms with a deletion strain (e.g., with NG2618 strain (Figure 8 and Figure 9),) and only if mutants were observed in the F1 generation, did the experiment proceed. When we observed mutants in the F1 generation after 2.5 to 3 days, we would transfer the worms that looked like WT hermaphrodites into new 60mm plates. After 2.5 to 3 days, the F2 generation was screened for worms that appeared WT, dpyUnc, or mutant. If all of these phenotypes were observed in the F2 generation, then the strain was said to be balanced.

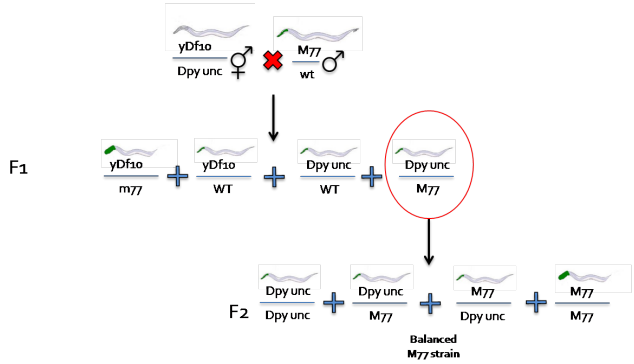

Figure 9: Schematic drawing for balancing the M77 allele with a deletion strain NG2618 or TY1353. Deletion (*yDf10*) over DpyUnc, which is the balancing strain, crossed with M77 over wt for that allele. In the F1 generation, we get deletion (*yDf10*) over M77 and if those are mutants, then we can get deletion over WT, which looks wt, we can get DpyUnc over wt or we can get DpyUnc over M77 all of which look-like WT except the mutants. Picking some of those WT and hoping to get the ones that are DpyUnc over M77, they produce DpyUnc over DpyUnc, DpyUnc over M77, M77 over DpyUnc, and M77 over M77 (which are mutants). DpyUnc over M77 and M77 over DpyUnc would appear WT. So, half of them will be WT, one quarter will be DpyUnc, and one quarter will be mutant. Since, DpyUnc over M77 and M77 over DpyUnc are genetically the same as their parents and look like WT, they will produce DpyUncs, wild types that are heterozygous (DpyUnc over M77 and M77 over DpyUnc), and they will give mutants. That is when the strain was balanced.

The following equipments were used often in this research project:

Equipment	Part number/Serial Number	Model number
Thermocycler-Eppendorf: Master cycler gradient	533140319	
Centrifuge 5403	540302110	
Microscope: LEICA MDG30 10446352	5514556	
BIO-RAD POWER*N*PAC 3000	1040	1655056
Gel Box: FILL LINE	224029	D3
Vortexer	16008	AB1A3201
Laboratory Stirrer	3.00498E+11	PC-410
Scale: Metter-Toledo GmbH	606	
VersaDoc Imaging System	590BR0046	Versa Doc Imaging System 300TM
Microwave: Montomery Ward	130453	
Nikon Eclipse TE2000-U		

Table 1: Data from crossing *C. elegans* male worms with BC4697 hermaphrodite worms: Plate number indicates the original plate number of the transferred BC4697 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. No mutant worms were observed in the F1 generation of the worms that successfully mated while 4 plates (2 in one trial and 2 in another trial (red)) revealed mutant worms in the F2 generation in those plates that mating had successfully occurred indicating that the BC4697 deletion complemented the M77 gene.

Plate number	Generation	Observations
1	F1	no *GFP*
2	F1	no *GFP*
3	F1	>
4	F1	>
5	F1	>
7	F1	>
1	F2	*
2	F2	*
1	F1	>
2	F1	>
3	F1	>
2	F2	*
3	F2	*
Key:	> = *GFP*, no mutants	*= mutants seen

Results

The *C. elegans* M77 mutant strain has phenotypic characteristics of a rounded, short, worm, similar to a dumpy phenotype, and it does not survive past the Larval 1 (L1) stage of development (Figure 1). In particular, the pharynx of M77 is vastly different than that of a WT worm. The pharynx of a WT worm has five anatomically distinct regions that can be easily distinguished from each other. These distinct WT pharyngeal regions include buccal cavity, procorpus, metacorpus, isthmus, and terminal blurb (Figure 1), regions which are almost indistinguishable in M77 mutant worms. Although M77 mutant worms develop the terminal bulb, their procorpus and metacarpus are indiscernible. Moreover, unlike the long-linear isthmus structure of a WT worm pharynx, M77 mutants have a much shorter isthmus region (Figure 2 and Figure 20 (A)). Finally, unlike fan-shaped functionary WT male tail, M77 mutants do not develop the sphericals of a male. In summary, there are obvious anatomically structure difference between the WT and M77 mutant worms.

Complementation analysis and further SNP mapping data of M77 mutants mapped the genetic defect to a region between -2.78 and -4.7 mapping unit (mu) on chromosome III, contrary to the previously reported M77 data. My colleague Andrew Ferrier suggested that the M77 mutant allele mapped close to the mor-1 gene, which also exhibits blunt phenotype; however, the recent data supports the mor-1 gene mapping outside this mu range. Still, there are many larval lethal genes that have been identified but not yet described between map units -2.78 and -4.7.

Complementation Analyses/Test

Previously, the M77 allele was mapped and reported by our lab using Chromosomal and SNP mapping techniques to chromosome III between the approximate region of -6 mu and -3 mu. To refine this region further, we utilized complementation testing, where we used several strains with known genetic deletions between the chromosomal regions of -1.46 and -12.6. These deletion strains included BC4637, BC4697, CB4681, MT690, MT696, MT699, NG2618, and TY1353.

First, we crossed the known BC4637 deletion strain with our M77 and screened the F1 and F2 for mutant worms. The BC4697 deletion strain contains the deficiency sDf121, and deletes the chromosomal region from -1.46006 to -12.6325 mu. BC4637 worms exhibit uncoordinated behavior and they are characterized as unc-32 (uncoordinated-32) animals. Moreover, they are also maintained by picking these unc-32 worms. We did not observe any plates that showed us indications as to whether mating had taken place between BC4637 and our strain of M77. Therefore, the data are inconclusive for this strain.

Second, we crossed the known BC4697 deletion strain with our M77 strain and screened the F1 and F2 for mutant worms. The BC4697 deletion strain, which contains the deficiency sDf121, and deletes the chromosomal region from -1.310 to -1.45 mu, and is phenotypically an uncoordinated strain. We did not observe mutant worms in the F1 generation of this strain crossed with M77 worms, but we observed mutants in 2 out of 7 plates, in which mating had taken place, and again in 2 out of 4 plates in the F2 generations (Table 1), showing the mating was successful. So, the BC4637 deletion data revealed complementation with the M77 mutant gene.

Third, we crossed the known CB4681 deletion strain with our M77 and again screened the F1 and F2 generation for mutant worms. The CB4681 deletion strain contains the deficiency nDf17, and covers the chromosomal region from -1.50094 to 2.12568 mu and exhibits dumpy (Dpy) heterozygotes (organisms having two different alleles of a particular gene) that segregate Dpy. These Dpy sterile worms produce only dead eggs. After cross of our M77 worms with this strain, we observed no mutant in the F1 generation whereas we observed mutants in 2 out of 5 plates. In which the mating had taken place (Table 2). This observation suggested to us that the gene of interest and the CB4637 deletion complemented each other.

Table 2: Data from crossing C. elegans male worms with CB4681 hermaphrodite worms: Plate number indicates the original plate number of the transferred CB4681 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. No mutant worms were observed in the F1 generation of the worms that successfully mated while 2 plates revealed mutant worms in the F2 generation in those plates that the mating had successfully occurred (red), indicating complementation between BC4681 deletion and the M77 gene.

Plate number	Generation	Observations
1	F1	>
1	F2	>
3	F1	>
12	F1	>
15	F1	>
18	F1	>
19	F1	>
20	F1	>
3	F2	*
20	F2	*
Key:	> = *GFP*, no mutants * = mutants seen	

Table 3: Data from crossing C. elegans male worms with MT690 hermaphrodite worms: Plate number indicates the original plate number of the transferred MT690 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. No mutant worms were observed in the F1 generation of the worms that successfully mated while 7 plates (6 in one trial and 1 in another trial (red)) revealed mutant worms in the F2 generation in those plates that mating had successfully occurred, indicating that the MT690 deletion complemented the M77 gene.

Plate number	Generation	Observations
1	F1	>
2	F1	>
3	F1	>
4	F1	>
5	F1	>
6	F1	>
7	F1	>
1	F1	>
2	F1	>
3	F1	>
4	F1	>
5	F1	>
6	F1	>
7	F1	>
1	F2	*
2	F2	*
3	F2	>
4	F2	*
5	F2	*
6	F2	*
7	F2	>
8	F2	*
1	F1	>
2	F1	>
1	F1	>
1	F2	>
2	F2	>
1	F1	>
1	F2	*
Key:	> = GFP, no mutants	*= mutants seen

Table 4: Data from crossing C. elegans male worms with MT696 hermaphrodite worms: Plate number indicates the original plate number of the transferred MT696 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. No mutant worms were observed in the F1 generation of the worms that successfully mated while 4 plates (1 in one trial, 2 in another trial, and 1 again in another trial (red)) revealed mutant worms in the F2 generation in those plates that mating had successfully occurred, indicating that the MT696 deletion complemented the M77 gene.

Plate number	Generation	Observations
1	F1	>
2	F1	>
1	F2	>
1	F2	*
2	F1	>
5	F1	>
6	F1	>
7	F1	>
1	F2	*
2	F2	*
1	F1	>
2	F1	>
3	F1	>
4	F1	>
1	F2	*
2	F2	*
3	F2	>
4	F2	>
Key:	> = *GFP*, no mutants * = mutants seen	

Fourth, we crossed the known MT690 deletion strain with our M77 strain and screened the F1 and F2 for mutant worms. The MT690 deletion strain contains the deficient nDf6, and covers the chromosomal region from -4.47033 to -7.01726 mu. MT690 exhibits heterozygotes are Unc and segregate a DpyUnc phenotype' DPY worms, and early larval lethals. This strain is maintained by picking Unc but non-Dpy worms. When we crossed this strain with M77 male worms, we observed again no mutant worms in the F1 generation. However in the F2 generation, we observed mutant worms in 5 plates in one trial, in which the mating had taken place, and in 1 plate in another trial, in which the mating had taken place successfully (Table 3). Therefore, the MT690 strain and the gene causing M77 mutation again complemented each other.

Fifth, we crossed the known MT696 deletion strain with our M77 strain and screened the F1 and F2 for mutant worm. The MT696 deletion strain contains the nDf12 deficiency, and covers the chromosomal region from -4.47033 to -5.52958 mu. MT696 deletion worms heterozygous worms that are Unc (Rubberband) and Egl (egg laying variant); segregate Unc, DpyUnc and dead eggs phenotype. Moreover, these worms exhibit variations in the stage of eggs laid, egg laying cycle, or egg laying in response to stimuli compared to control and are maintained by picking Uncs. When we mated this strain with our M77 strain, we observed no mutant worms in the F1 generation. However in the F2 generation, we observed mutant worms in 1 out of 3, 2 out of 7, and 1 out of 4 plates, in which the mating had taken place in the F2 generation (Table 4). These data also suggested to us that the gene of interest and the MT696 deletion complemented each other.

Sixth, we crossed the known MT699 deletion strain with our M77 strain and screened the F1 and F2 for mutant worms. The MT699 deletion strain is characterized by the nDf15 deficiency, and covers the chromosomal regions from -4.47033 to -7.01726 mu. This strain exhibits heterozygotes that are Unc and segregate Unc, DpyUnc, and production of dead eggs. In order to maintain this strain, we picked Unc worms. When we crossed this strain with the strain of interest, again we observed no mutant worms in the F1 generation. However, we also observed no mutant worms in the F2 generation, in those plates that we identified as the ones in which the mating had taken place (Table 5). We observed high male frequency in this deletion strain and we tried many times to get pure hermaphrodite by picking L1 worms but it was not successful. Next, we carried a reverse cross, in which we mated M77 hermaphrodites with MT699 male worms, and then transferred those mated M77 hermaphrodites into individual new plates. In addition to screening the F1 and F2 generation for mutants, we particularly screened the F1 for male worms and F2 generation to look for a quarter of non- green worms (worms with no GFP protein), as it was an indication of the fact that mating had successfully occurred. Although we observed in the F1 generation in some plates, however, neither did we see males in the F1 generation nor the non-green worms (Table 6). Therefore, the data for this strain is inconclusive.

Table 5: Data Data from crossing C. elegans male worms with MT699 hermaphrodite worms: Plate number indicates the original plate number of the transferred MT699 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. No mutant worms were observed in the F1 generation of the worms or in the F2 generation because no successful mating occurred between the MT699 deletion strain in our M77 strain. Therefore, the results are inconclusive for this strain.

Plate number	Generation	Observations
1	F1	>
2	F1	>
3	F1	>
4	F1	>
1	F2	>
2	F2	>
3	F2	>
4	F2	>
Key:	> = *GFP*, no mutants * = mutants seen	

Table 6: Data from crossing *C. elegans* hermaphrodite worms with MT699 male worms: Plate number indicates the original plate number of the transferred MT696 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. Percent of mutant verses non-mutants worms in the F1 generation were counted and can be seen in the table. All worms were green (with GFP protein) in both the F1 and F2 generations, which indicated that the mating did not successfully occurred between the MT699 and the strain of interest, therefore no conclusion can be drawn for this strain yet.

Plate #	Generation	# of Mutants	#of Wildtypes	% mutants	% Wildtypes	Worms with GFP
1	F1	0	1			all
2	F1	217	401	35.1	64.9	all
3	F1	0	0	0.0	0.0	all
4	F1	0	0	0.0	0.0	all
5	F1	169	316	34.8	65.2	all
6	F1	62	94	39.7	60.3	all
7	F1	153	310	33.0	67.0	all
8	F1	151	410	26.9	73.1	all
9	F1	57	284	16.7	83.3	all
10	F1	0	1			all
11	F1	0	0	0.0	0.0	all
12	F1	66	306	17.7	82.3	all
13	F1	0	0	0.0	0.0	all
14	F1	0	0	0.0	0.0	all
15	F1	26	88	22.8	77.2	all
1	F2					all
2	F2					all
3	F2					all
4	F2					all
5	F2					all
6	F2					all
7	F2					all
8	F2					all
9	F2					all
10	F2					all
11	F2					all
12	F2					all
13	F2					all
14	F2					all
15	F2					all
Key:	0 = no worms in the plate were observed Blank = only 1 transferred hermaphrodite or the mom was observed					

Seventh, we crossed the known NG2618 deletion strain with our M77 strain and screened the F1 and F2 for mutant worms. The NG2618 deletion strain contains the yDf10 deficiency that deletes the chromosomal regions from -2.78397 to -5.22301 mu and produces progeny that are WTs, Dpy, and sterile worms with dead eggs. NG2618 strain was derived from strain TY1353 and it grows pretty slowly but seems more stable than TY1353, which gives lots of sterile worms. Unlike the previous deletion strains, when NG2618 worms were crossed with M77 strains, we observed mutant worms in the F1 generation worms in one trial, suggesting no complementation between the gene of interest and the NG2618 deletion, followed by 3 plates of possible F2 generation in another trial where the mating had worked. Moreover, possibly in the F2 generation, we also observed what looked like mutant worms in one additional plate (Table 7). This was contrary to the earlier experiment that suggested complementation between the two genes. After an assessment of the original deletion strain, it was established that the deletion had broken down at some point after the first experiment, meaning that the deletion was no longer there. After ordering a new strain of NG2618 deletion strain from CCG, we repeated the experiment and we observed mutant worms in the F1 generation in 2 plates, in which the mating had taken place. In the first plate, roughly 5 out 18 worms and in the second plate roughly 4 out 12

Table 7: Data from crossing C. elegans male worms with NG2618 hermaphrodite worms: Plate number indicates the original plate number of the transferred NG2618 hermaphrodite after mating with M77 male worm and generation shows the successive generations of that hermaphrodite. 9 plates (8 plates and 1 plate that looked-like with mutants (red)) revealed mutant worms in the F1 generation while there were no mutants observed in the F2 generation of plates where the mating had successfully occurred, except when the strain broke down. Therefore, the results on the table show that the BC4697 deletion did not complement the M77 gene.

Plate number	Generation	Observations
1	F1	*
1	F1	>
2	F1	>
3	F1	>
4	F1	>
1	F2 pos	*
2	F2 pos	possible *
3	F2 pos	*
4	F2 pos	*
1	F1	>
2	F1	>
3	F1	>
1	F2	*
2	F2	*
2	F1	>
1	F2	>
1	F1	* (roughly 5 out 18 worms mutants)
2	F1	* (roughly 4 out 12 worms mutants)
Key:	> = *GFP*, no mutants * = mutants seen	

Table 8: Summary of the total number of crosses between the deletion strains and M77 strain: Out of 934 crosses of M77 worms with deletion worms, 54 crosses resulted in progeny and of those 27 of them were successful crosses due to successful mating.

Strain	total # of times crossed	# of plates the mating had taken place	# of plates the mating was successful	% of plates in which mating had taken place	% of plates, in which mating was successful	% of plates in which mating was successful out of plates that mating had taken place
BC4637	40	0	0	0.0	0.0	0.0
BC4697	90	8	4	8.9	4.4	50.0
CB4681	40	7	2	17.5	5.0	28.6
MT690	230	18	7	7.8	3.0	38.9
MT696	230	10	5	4.3	2.2	50.0
MT699	40	0	0	0.0	0.0	0.0
NG2618	224	11	9	4.9	4.0	81.8
Ty1353	40	0	0	0.0	0.0	0.0
Total	934	54	27			
Average of the plates, in which mating had successfully occurred						49.9

worms were mutants (Table 7). These data as were expected, suggested to that the gene of interest and the NG2618 deletion do not complement each other.

Finally, we crossed the known TY1353 deletion strain with our M77 strain and screened the F1 and F2 for mutant worms. The TY1353 deletion strain, from which the NG2618 deletions strain was derived, is also deficient for yDf10 allele and covers the chromosomal region from -2.78397 to -5.22301 mu. TY1353 worms exhibit the following phenotypic characteristics: heterozygotes are Unc-93 and segregate more Unc-93, yDf10 homozygotes (organisms with two identical alleles of a particular gene and thus they breed true for the particular characteristic), which produce dead eggs, and Unc-93 Dpy-17 homozygotes (young dpy-17 larvae are easily recognizable as abnormal spindle-shaped things). This strain is fairly difficult to maintain and yDf10 apparently causes semi-sterility, while unc-93 is Egl and difficult to mate into. Some homozygotes are laid, but the majority of them remain inside the mother. When we crossed this strain with our M77 worms, we observed again no mutant worms in the F1 generation. However, the mating never was successful with this strain.

As can be seen in Table 8, for example, 230 MT690 worms were crossed with M77 worms, from which only 18 of them identified to have had mated, and 7 of those that actually successfully mated. When green worms were observed in the F1 generation of the crossed deletion strain hermaphrodite, it was said that the mating had occurred since the deletion strains had no myo-2::GFP protein tagged in their pharyngeal cells. The successful mating was scored based on observation of green worms, non-green worms, male worms, and mutant worms either in the F1 or F2 generations. Once, the mating had occurred, it was generally about 50% successful (Table 8).

SNP interval mapping

Usually, SNP mapping is carried in two phases, the chromosome mapping, which identifies the relevant chromosome and rough position of the mutant gene, and interval mapping, which narrows the mutant gene in an interval between two SNPs, and can also be used repeatedly to refine the position of the gene of interest once its location has been roughly determined by chromosome mapping. More specifically, the difference between the two is that chromosomal mapping seeks to determine the genotype of pooled animals while the interval mapping seeks to determine the individual's genotype. However, both phases share similar procedure. Since, the chromosomal location of the M77 allele was roughly determined by our lab previously to chromosome III between loci -6 and -3, we focused on the interval mapping to refine this region further in order to determine the identity of the gene of interest that causes the M77 phenotype.

In order to refine the chromosomal region of the gene of interest, we chose loci (+4 and -7 respectively) and looked into the recombination frequency between the M77 mutant (Bristol, N2) and Hawaiian DNA when we obtained gel electrophoresis picture. In evaluating the gel, we observed DNA band patterns according to the table of DraI SNP primers outlined by Davis et. al., 2005.

The interval map of +4 showed 2 lanes of recombination in which both the N2 355 band and Hawaiian 497 bands were observed (Figure 11). The interval map also showed 7 lines (14 chromosomes) with pure N2 DNA patterns, indicating no recombination and 88 lines with either missing DNA or lines that were unreadable. The recombination frequency at map unit +4 was calculated to be 0.125%, which suggested that the gene of interest lays ±12.5 mapping unit from +4 chromosomal III locus (Table 9).

The interval band of -7 contained 6 recombinations, in which both the N2 DNA band 239 and Hawaiian band 196 were seen. There were also 33 lines in which only N2 DNA was seen, indicating no recombination and 57 lines either with no DNA or unreadable (Figure 12). The recombination frequency of map unit -7 worked out to be 0.0769%, suggesting that the gene of interest might be placed ± 7.69 mu from -7 chromosomal III locus (Table 9). In another trial, the interval map for -7 contained 1 recombination, in which both the N2 DNA band at 239 and Hawaiian band 196 were observed, while 11 lines in which only N, and 84 lines with no DNA or unreadable were seen (Figure 13). The recombination frequency for this trial worked out to be 0.0461%, suggesting the gene of interest is ± 4.61 mu from -7 (Table 9). The average of the two trials ((7.69+4.61)/2) was ±6.15 mu from the -7 chromosomal locus. It is worth mentioning that the interval mapping of -7 data varied for all the other trials and they were inconclusive. In two of the trials using DNA from the same sets of mutants, all of the DNA bands seen on the gel picture seemed to be at 239, implying that all the bands are of N2 DNA and therefore no recombination (Figure 14 and Figure 15). Finally, in another two trials, using the DNA from the same sets of mutants, again all the bands seemed to be N2 DNA pattern, thus suggesting no recombination, and the other was unreadable (Figure 16 and Figure 17).

Using the data of the complementation analyses, we constructed a schematic drawing of the location of the gene of interest (Figure 10). After mapping data from the cross between our strain M77 and the deletion strains, the gene of interest maps roughly at the chromosomal region between -3.10 and -4.47 mu (Figure 10).

Table 9: Interval Mapping Data for +4 and -7 Chromosomal Luci. Each line represents two chromosomes: N2 lines are multiplied by 2 since they are homozygous for the mutation and therefore carry M77 allele in each chromosome. The total number of chromosome is obtained by adding the total number of homozygous (N2 DNA lines x 2) to total number of heterozygous (N2 DNA + Hawaiian DNA lines or lines with recombination). The recombination frequency is calculated by dividing the number of recombinants by the total number of chromosomes, which is in turn multiplied by 100 to obtain recombination frequency percentages.

Interval Locus	Lanes with Recombination (N2 DNA +Hawaiian DNA)	Lines with only N2 DNA	Total # of Chromosomes	Recombination Frequency %	± mu
+4	2	7	18	12.50%	12.5
-7	6	33	78	8.33%	8.33
-7	1	11	24	4.35%	4.35

Mapping of the *m77* Gene Location

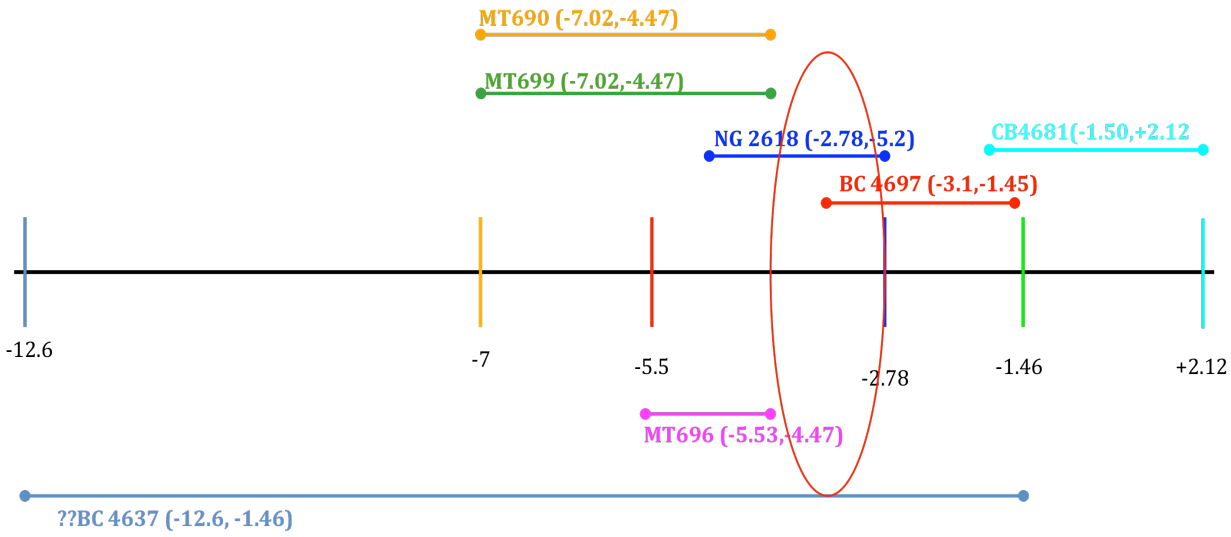

Figure 10: Schematic construction of rough chromosomal location of the M77 gene using complementation analyses data: The black horizontal line represents chromosome III, and vertical lines (orange, red, blue, green, and blue) represent either the start or the end of the deletion region. According the complementation analyses data, the M77 gene maps between the region of -3.10 and -4.47.

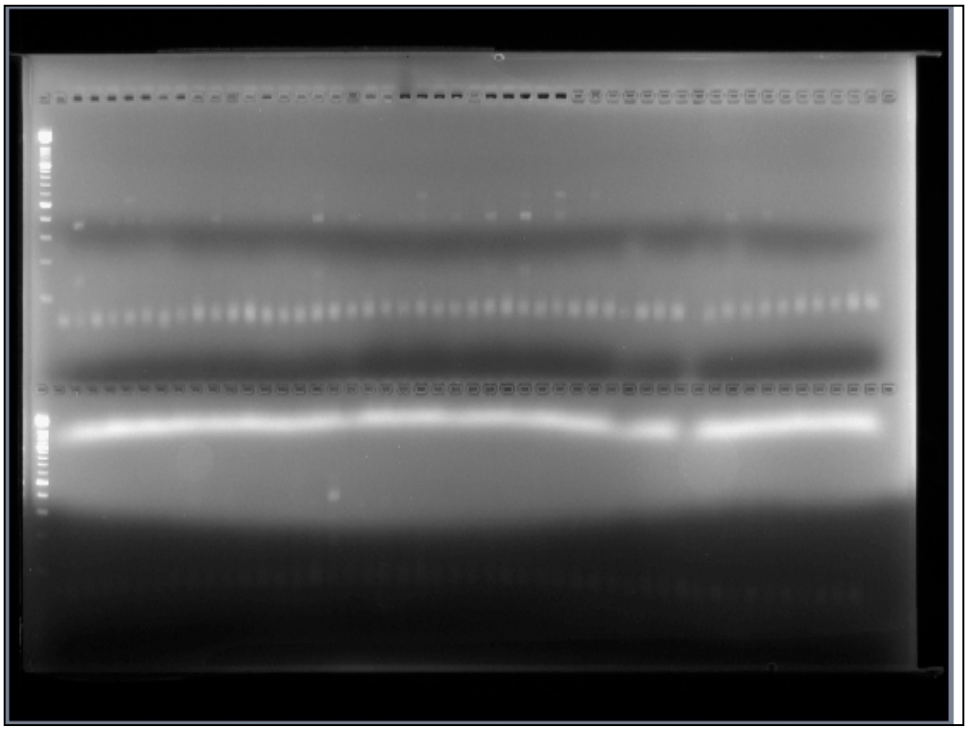

Figure 11: Interval mapping of M77: 2.5% agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. The pattern for heterozygous (recombinant) is different than the pattern for homozygous. The recombination frequency of interval map of map unit +4 on chromosome III calculated for this gel was 12.50%.

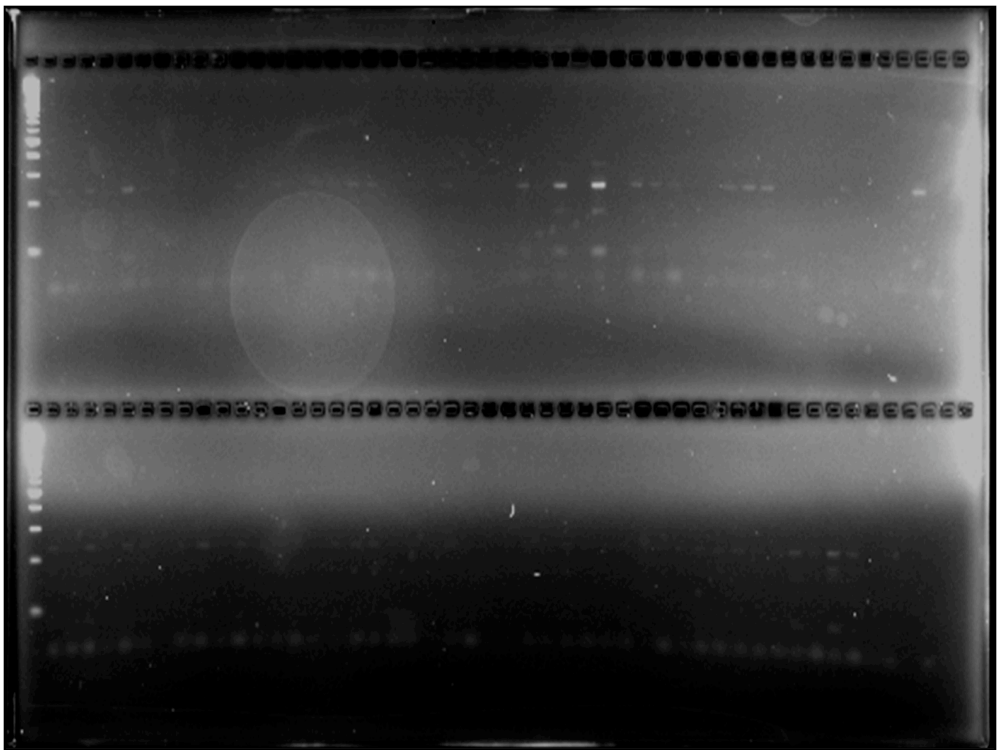

Figure 12: Interval mapping of M77. 2.5% agarose gel electrophoresis of interval maps: Each lane represents one mutant genotype. The pattern for heterozygous (recombinant) is different than the pattern for homozygous. The recombination frequency of interval map of map unit -7 on chromosome III worked out for this gel was 8.33%.

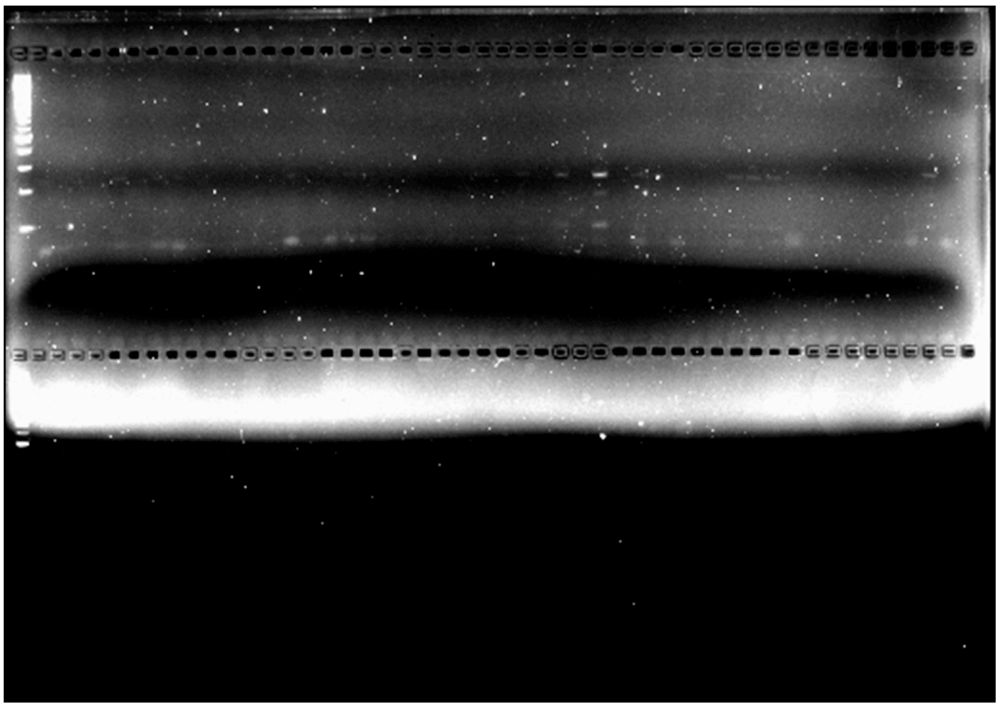

Figure 13: Interval mapping of M77: 2.5% agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. The pattern for heterozygous (recombinant) is different than the pattern for homozygous. The recombination frequency of interval map of map unit -7 on chromosome III worked out for this gel was 4.35%.

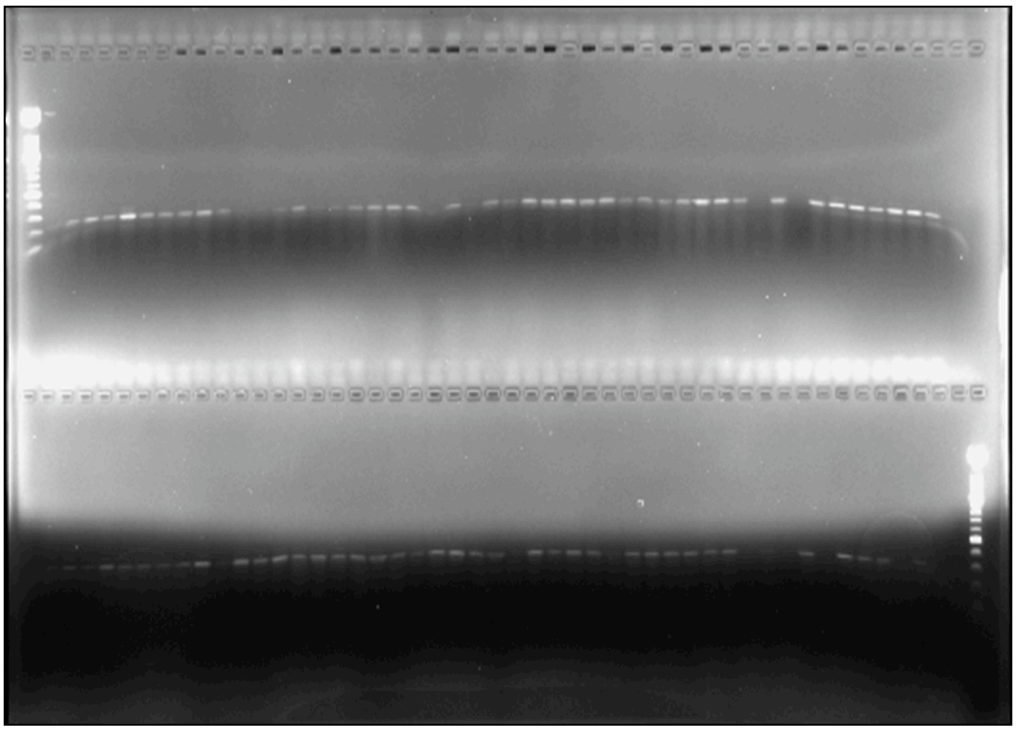

Figure 14: Interval mapping of M77: 2.5% agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. Only N2 DNA patterns seen in this gel and no recombinant pattern seen. The recombination frequency of interval map of map unit -7 on chromosome III suggested by this gel is 0%.

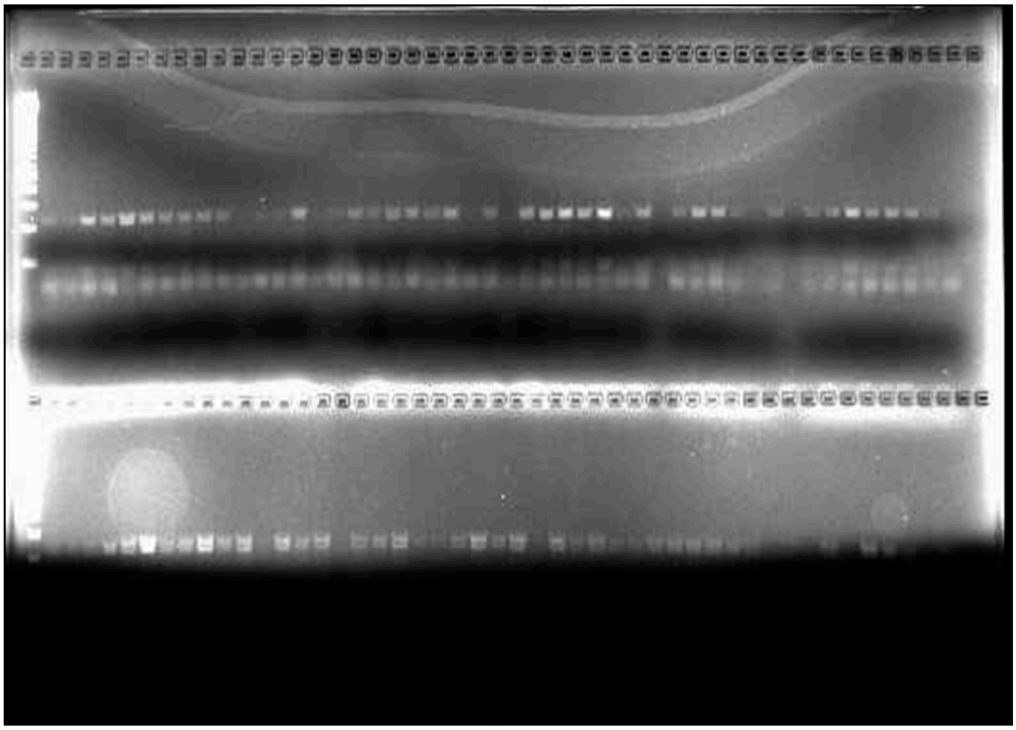

Figure 15: Interval mapping of M77: 2.5% agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. Only N2 DNA patterns recognized in this gel and no recombinant pattern observed. The recombination frequency of interval map of map unit -7 on chromosome III suggested by this gel is also 0%.

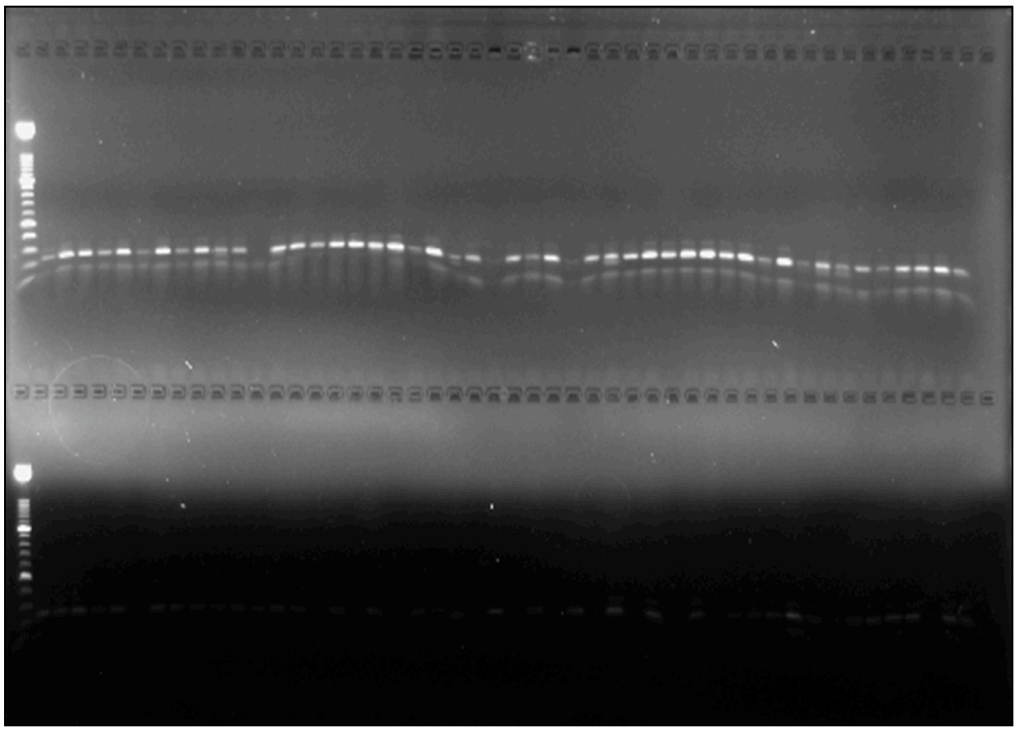

Figure 16: Interval mapping of M77: 2.5% agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. Only N2 DNA patterns seen in this gel and no recombinant pattern seen. The recombination frequency of interval map of map unit -7 on chromosome III suggested by this gel is 0%.

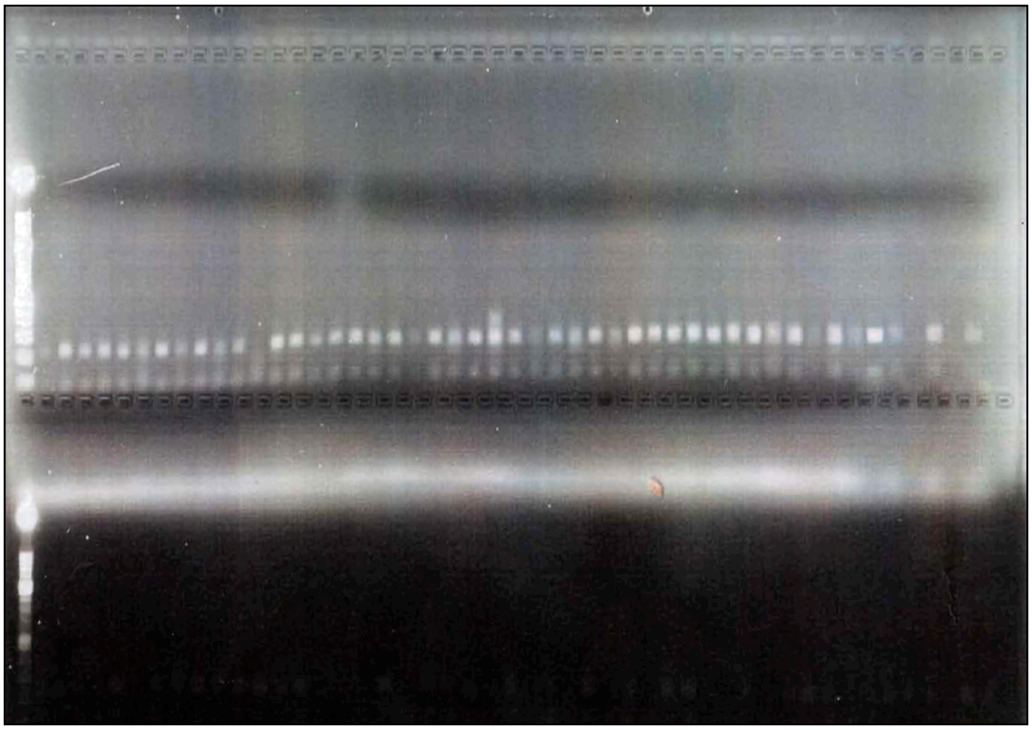

Figure 17: Interval mapping of M77. 2.5% agarose gel electrophoresis of interval maps: Each lane represents one mutant genotype. Only N2 DNA patterns seen in this gel and no recombinant pattern seen. The recombination frequency of interval map of map unit -7 on chromosome III suggested by this gel is 0%.

Antibody Staining of M77 worms

The myo-2::GFP and antibody stain worked for KT14, KT20, and KT36. Myo-2::GFP antibody worked for all the worms (Figure 18). The KT14, which recognizes the basement membrane, worked as well where mutant worm (Figure 18 (A')) appeared to be missing the basement membrane in the pharynx, while the WT worm is not (Figure 18 (A)). On the other hand KT20 and KT36 that recognize the apical side of pharynx, seemed to be the same for both the WT worm and the mutant worm (Figure 18 (B, B', C, and C'). No antibody stained worms were observed for KT16, MH4, and MH27 antibodies.

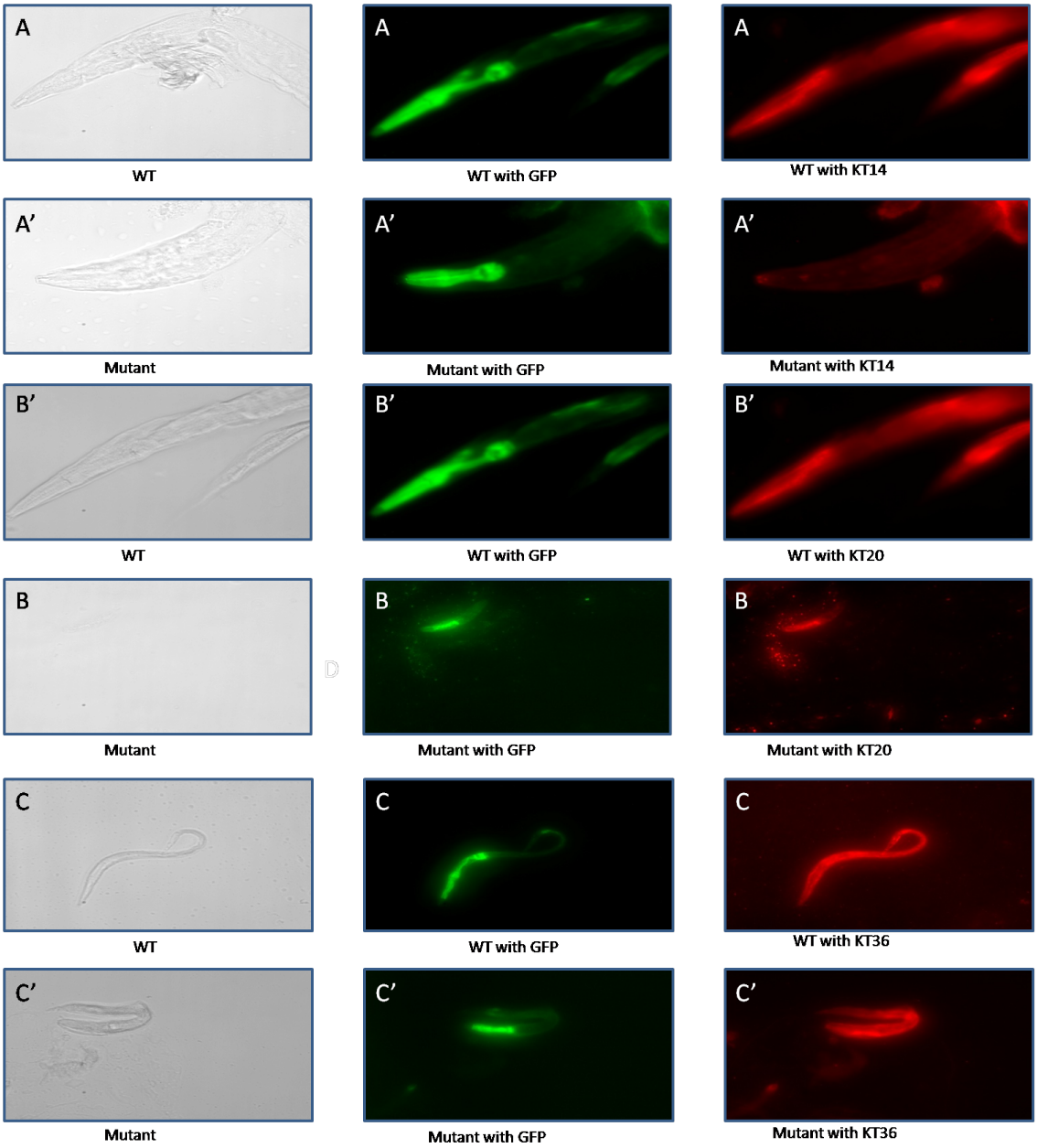

Figure 18: Pharynx-specific antibody staining of M77 worms: KT20 and KT36 recognize the apical side of pharynx and KT14 recognizes basement membrane. From left to right, (A) WT worm, WT with myo-2::GFP, and WT with KT14 antibody. (A') mutant worm, mutant with myo-2::GFP, and mutant with KT14 antibody. (B) WT worm, WT with myo-2::GFP, and WT with KT20 antibody. (B') mutant worm, mutant with myo-2::GFP, and mutant with KT20 antibody. (C) WT worm, WT with myo-2::GFP, and WT with KT36 antibody. (B') mutant worm, mutant with myo-2::GFP, and mutant with KT36 antibody.

Balancing the M77 allele

Preservation of a mutant strain is an essential part of C. elegans genetics. The recessive lethal mutant strains, like M77, cannot be maintained as homozygotes and heterozygous can be lost easily through segregation, except if there are methods to identify them. In order to keep heterozygotes genotype, scientists are required to choose heterozygous worms and physically observe the mutant phenotype in their progenies. This is a tedious and challenging task because, for example, the self progenies of a M77 heterozygotes are either homozygotes with lethal mutation (let+/let+), which arrest at L1 sage of the development, heterozygotes for lethal mutation (let+/+), which phenotypically appear like WTs, and WTs that cannot be distinguished phenotypically from the heterozygotes. Therefore, many phenotypically WT worms must be transferred to preserve a strain, like M77. It should be apparent from this that any large scale isolation or studies of lethal mutations, like M77, requires more effective means of preservation to prevent loss of the strain. Moreover, it will require a clear and easy way for a researcher to distinguish between the homozygotes for mutation, heterozygotes for mutation, and WT worms. Balancing the strain is what provides both of these aforementioned tasks.

Therefore, the second part of this project was to genetically balance the M77 allele or strain. Again, the reason for this is to preserve the strain to prevent the mutant allele from being lost as the population reproduces. Furthermore, once the strain is balanced, it will also be easy to work with, meaning one will know which worms will lead to mutants. The way a researcher can balance a particular mutant strain is by crossing it with a deletion strain, called the balancer, which very closely maps to the mutation of the desired strain, and then screen the F1 generation of cross for mutants. If the mutant worms are observed, then the desired strain can be balanced. But, if the mutants are not seen in the F1 generation means that the researcher cannot balance the desired strain and it just tells him/her that the experiment worked. However, when mutants are observed in the F1 generation, then WT appearing worms are transferred into new plates, and then their progenies are screened for the expected phenotypes of the balancer and the desire strain when they are crossed. Finally, if those expected phenotypes are observed, then the desired strain can be said to have been genetically balanced.

It should be apparent by now that we used complementation analysis procedure in order to balance the strain of interest. Since, the balancing only works when there are mutants in the F1 generation of the cross between a desired strain and a deletion strain, meaning when there is no complementation, to balance our M77 strain, we used the NG2618 deletion strain, as it was the only strain that showed no complementation in the F1 generation. In short, we crossed deletion (yDf10) over DpyUnc, which is the balancing strain, with our M77 over WT for that allele (Figure 9). We observed mutants in the F1 generation and the rest of the worms showed WT phenotype. Observing mutants in the F1 generations suggested that the gene of the interest is in the deletion region, which was what we needed. Then, we picked some of the WT exhibiting phenotypic characteristics worms into new plates hoping that some of them had the DpyUnc over M77 genotype. Since, all the worms, except the mutants, exhibited WT phenotype, we did not know if we picked the worms with the right phenotype until we observed the phenotypes of their progenies. Next, we again transferred the F1 generation of the transferred worms, and in the F2 generation, we observed mutants again when they produced progenies. In fact in the F2, we actually observed in one of the plates all the phenotypes necessary to suggest that the strain was balanced. We observed about half of the worms mutants and Dpys and the other half WT worms. We also observed that the Dpy worms and mutant worms are indistinguishable under microscope until L1 stage. Both, Dpys and mutants showed similar phenotypes. The only difference between the two was that Dpys survive the L1 developmental stage, because they can consume food, but M77 mutant worms cannot survive the L1 developmental stage (Figure 17).

Despite realizing that the phenotype of the Dpy worms until L1 developmental stage is similar to M77 mutant worms, we picked many individual worms and observed their progenies' phenotype to confirm that the strain was balanced. We expected that each WT worm that we pick should produce a quarter of mutant worms, a quarter Dpy worms, which are similar in phenotype to our mutant worms until L1 stage, and two quarters of heterozygotes, which phenotypically appear like WT worms. Since the M77 mutant worms and Dpy worms could not be phenotypically distinguished, we expected two quarters (50%) of the progenies to be mutants. The average of worm counts that we performed was 37 % (M77 mutant and Dpy) worms with mutant phenotype and 63% worms with WT phenotypes (heterozygotes) (Table 10).

Table 10: Worm Count of Balanced M77 Worms: Worm count of mutant worms compared to the wildtype worms to confirm that the M77 strain was balanced. Please see below for the continuation of Table 10 data.

Plate #	Mutants	Wildtype	% Mutants	% Wildtype
1	84	123	40.6	59.4
2	55	99	35.7	64.3
3	112	221	33.6	66.4
4	87	115	43.1	56.9
5	79	201	28.2	71.8
6	33	51	39.3	60.7
7	61	112	35.3	64.7
8	42	61	40.8	59.2
9	88	155	36.2	63.8
10	115	138	45.5	54.5
11	29	51	36.3	63.8
12	43	63	40.6	59.4
13	58	124	31.9	68.1
14	62	97	39.0	61.0
15	127	166	43.3	56.7
16	119	251	32.2	67.8
17	76	131	36.7	63.3
18	81	101	44.5	55.5
19	62	83	42.8	57.2
20	93	111	45.6	54.4
21	53	77	40.8	59.2
22	45	103	30.4	69.6
23	65	98	39.9	60.1
24	33	47	41.3	58.8
25	29	67	30.2	69.8
26	64	81	44.1	55.9
27	580	900	39.2	60.8
28	796	900	46.9	53.1
29	196	328	37.4	62.6
30	460	720	39.0	61.0

Table 10': Continuation of Table 10 data: Worm Count of Balanced M77 Worms: Worm count of mutant worms compared to the wildtype worms to confirm that the M77 strain was balanced. On average 37% mutant worms and 63% wildtype worms were observed in each screened plate, containing balanced M77 worms.

Plate #	Mutants	Wildtype	% Mutants	% Wildtype
31	27	62	30.3	69.7
32	11	32	25.6	74.4
33	7	9	43.8	56.3
34	30	35	46.2	53.8
35	13	21	38.2	61.8
36	9	17	34.6	65.4
37	5	14	26.3	73.7
38	8	18	30.8	69.2
39	4	11	26.7	73.3
40	5	19	20.8	79.2
41	31	55	36.0	64.0
42	36	61	37.1	62.9
43	29	53	35.4	64.6
44	44	66	40.0	60.0
45	59	116	33.7	66.3
46	28	38	42.4	57.6
47	37	89	29.4	70.6
48	15	31	32.6	67.4
49	19	27	41.3	58.7
50	33	55	37.5	62.5
51	31	63	33.0	67.0
52	43	77	35.8	64.2
53	27	33	45.0	55.0
54	28	37	43.1	56.9
55	39	98	28.5	71.5
56	42	64	39.6	60.4
57	27	79	25.5	74.5
58	29	35	45.3	54.7
59	19	41	31.7	68.3
60	53	69	43.4	56.6
Average	76.92	120	37.0	63.0
SD			6.2	6.2

Discussion

The most important findings of this research project were that we were able to further narrow down the chromosomal region of the M77 mutant gene from a previously reported -3 to -6 mu range to between -3.10 and -4.47 (which is within 1.37 mu), and we genetically balanced the M77 allele with a balancer strain NG2618. Furthermore, one of the most interesting findings of this research was that 7% ethanol treatment rescued the M77 mutant worms.

The *C. elegans* pharynx has emerged as one of the most valuable simple models for researchers to study and understand the genetics of cell patterning and morphogenesis (Mango, 2007). The goal of our lab is to use this organ to identify genes responsible for mutated gene phenotypes in order to contribute to the construction of an accurate genetic map indicating gene mechanisms for normal C. elegans pharyngeal development. In this research project, we focused on homozygous recessive mutant strain, M77, which exhibits blunt/shortened pharyngeal phenotype. We used complementation analysis, genetic mapping, and anti-body stain techniques in this study to determine the genetic and molecular cause of this blunt/short pharynx phenotype resulting from a mutation in a previously unmapped gene. We hypothesized that this mutant pharynx might have resulted from an abnormal morphogenesis, a vital developmental stage, which is marked by cellular movement and that ensures the proper differentiation and growth of specialized tissues and organs, like the pharynx, during organogenesis.

Morphogenesis and M77 mutant phenotype

In this project, we believe the cause of our M77 blunt/shortened phenotype may lie somewhere in one of the morphogenesis phases, whether it is the malfunction of the reorientation, epithilization, or the contraction phase, we currently do not know. As stated earlier, morphogenesis is a crucial developmental step for an organism because it is responsible for the formation of an organism's structure. More specifically, it is responsible for the differentiation and growth of tissues and organs, like the *C. elegans* pharynx. Therefore, failure in any steps of morphogenesis can lead to tremendous developmental abnormalities in the structural parts of an organism.

The M77 blunt phenotype

Our M77 mutant strain exhibits abnormalities in the metacarpus and procorpus. In addition, it displays a decreased isthmus length compared to the WT. The M77 mutant worms only grow until the L1 developmental stage and then die at that stage. The cause of M77 phenotype, as aforementioned, might be some kind of malfunction of cells during morphogenesis, which in turn may affect the pharynx structure and thus preventing C. elegans embryos from surviving past L1 developmental stage. Since the pharynx functions as a means of obtaining food and nutrients for the worm, it is most likely that the defects in M77 pharynx prevent the worms from properly consuming bacteria. This in turn may lead to the starvation and death of the worms after they are hatched from eggs at L1 developmental stage. The inability of consuming food and arresting due to starvation was shown by my colleague, Andrew Ferrier, who also studied the M77 worm strain. Additionally, Alexandra Charron, studied M136 and M138 worm strains, which exhibited extreme pharyngeal morphological defects by conducting a visual feeding assay. They both fed for two hours, the WTs and mutants, with OP50 E. coli strain that contained detectable Fluoresbrite Polychromatic 0.5 micron Microspheres beads diluted in water. When Ferrier (2009) and Charron (2010) examined them under Zeiss Axiovert 100 microscopy, they observed the marker beads in WTs and not in the mutants. Also, the larval lethality may just very well be caused by another mutation in the M77 strain because the EMS by which M77 mutation was caused may cause more than one point mutation or a small deletion of a chromosome segment in a strain(Charron, 2010; Yandell, Edgar, & Wood, 1994).

Narrowing/Refining Chromosomal Region of M77 gene with interval mapping and complementation test

Using the combination of chromosomal and interval mapping of single nucleotide polymorphism mapping, we have previously mapped the mutant allele to chromosome III roughly between the region of -3 and -6 (Ferrier, 2009). In this study, we continued using the interval mapping to further refine this chromosomal locus. Despite having difficulty of reading the interval map data from several gel electrophoresis pictures, the few that we were able to read, placed our gene of interest between chromosomal loci -4 and -6, which is consistent with what we have previously reported (Table 9 and Figure 8).

Using complementation test, we narrowed down the chromosomal region of the mutated gene to between map units -3.10 and 4.47 (Table 1-8; Figure 8). Sometimes, there are mutations in various genes that exhibit similar phenotypic characteristics and it is not only very difficult to distinguish between them but it is also very challenging to determine as to how many genes one is dealing with until their genetic loci are identified. Complementation analysis/test, which is a genetic test, allows scientists to identify these genes loci and thereby distinguish between them as well as determine how mutable each of those genes is (Yook, 2005). More specifically, it is a simple test of identifying whether two mutations that produce a similar phenotype are caused by the same allele (Yook, 2005). Therefore, by utilizing complementation test, we were able to further refine the location of M77 mutation to between chromosomal regions of -3.10 and -4.47 mu.

To assess the success or failure of complementation test, we used the following criteria when we screened the F1 generation of each crossed M77 worms with the deletion worms. When GFP-expressing worms and male worms, which demonstrated the success of mating, as well as mutant worms, were observed in the F1 generation of deletion strains crossed with heterozygous M77 male worms, it was said that the gene of interest and the deletion do not complement each other and therefore indicating that the mutant allele and the deletion allele were of the same gene. However, if only green worms, male worms but not mutant worms were observed in the F1 generation of the crossed worms, then it was said that our gene and the deletion complement each other. Thus, this indicates the M77 allele and the deletion allele are not of the same gene. Finally, if green worms were not observed at all, it was said that the mating was unsuccessful or failed and therefore was not used for complementation analyses.

Conducting Complementation tests were a bit challenging primarily due to getting deletion strains ready to go while obtaining heterozygous M77 worms. It was even more challenging to mate the deletion strains with our M77 strain successfully due to a variety of reasons, such as deletions worms being uncoordinated and preventing male worms from successful mating. Also, it might be that the M77 mutant gene might have a pleiotropic effect, where it causes shortened/blunt phenotype as well as affecting the mating behavior of male worms. Furthermore, it might be possible that one of the deleted genes in the deletion strains, where many genes are deleted from a portion of one of chromosome III in a worm, also have a pleiotropic effect that alters the mating behavior of worms. Therefore, it took us to cross many worms for each deletion strain with our strain to get a few plates that the mating actually had occurred.

Search for the gene mutation producing the short pharynx phenotype

After refining the chromosomal region of the gene that causes M77 blunt/shortened pharyngeal phenotype, we used the C. elegans wormbase website, which is a great database that shows all known mapped genes on all six C. elegans chromosomes, to determine whether there is any known gene exhibiting phenotypes like M77 mutation gene. More specifically, we searched for genes within -3.10 and -4.47 chromosomal map unit that have phenotypes similar to ones we have seen with the blunt/shortened pharynx M77 mutant worms. We have previously thought the gene that causes M77 mutation might be mor-1, which initially was determined by two point mapping of mor-1 and dpy-18, to be on chromosome III located in -3.81 ± 3.91, latter revised by the wormbase to be at the chromosomal III, -8.95 ±6.58 mu, and now revised again to chromosome III, -9.23 +/- 6.596 (Wormbase WS210, 2010; Ferrier, 2009). mor-1 genetic position is still under investigation because it has not yet been cloned and its genetic position is not well ordered as well (Wormbase WS210, 2010). Therefore, given the nature of the mor-1 genetic position not being well ordered yet, it is still a possible candidate for the gene that causes the blunt phenotype. However aforementioned genetic positions for mor-1 are not consistent with our complementation analysis data, which indicates that the M77 mutant allele is located between the region -3.1 and 4.7 mu. Therefore, we searched within this new refined region for another likely candidate gene that demonstrates similar pharyngeal phenotypes to our M77 mutant strain.

Hunting for the genes within -3.10 and -4.47 mu, we quickly realized that there are many genes within this region and many of them have not been described yet (Figure 19). However, there are not any known genes in this region that show a similar phenotype to that of our M77 mutants' phenotype. Since we know that M77 mutant worms are L1 lethal, which means the mutation causes death at L1 developmental stage, we can speculate that if it is one of these genes (between the region -3.10 and -4.47 mu) that causes M77 phenotype, then it is most likely that this gene might be one of these Lethal genes, such as let (lethal)-710, let-796, let-812, or any other of lethal genes in the region.

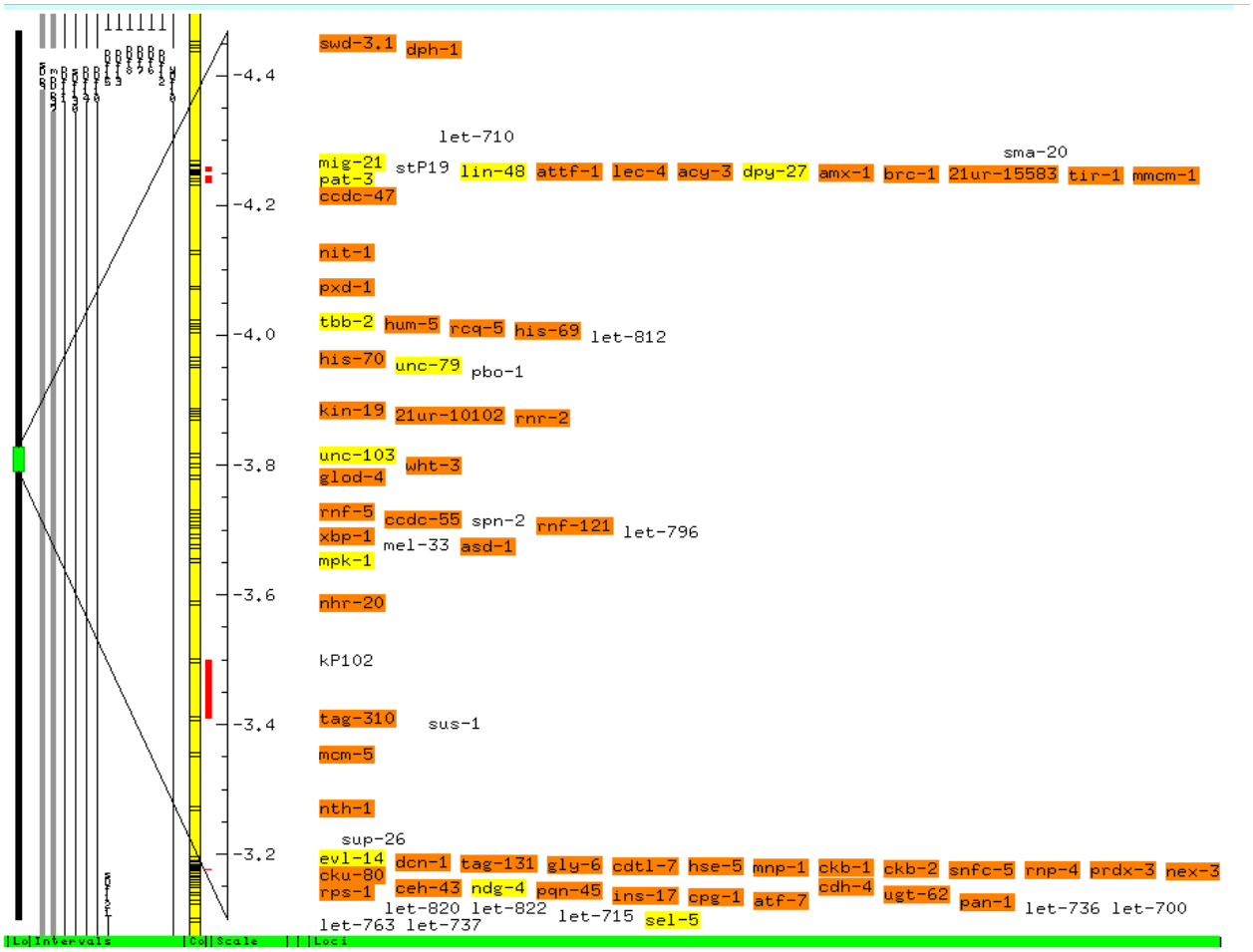

Figure 19: Genetic map constructed on Wormbase illustrating genes between the regions -3.10 and -4.47 mu on chromosome III: The black line indicates C. elegans chromosome III and the green region on the black line, indicates the region -3.10 and -4.47 mu. Genes highlighted in orange or yellow have been described and genes that are not highlighted have not been well described yet.

Given the blunt/short phenotype of M77 mutant worms and the fact that dpy-27 gene has been determined to be located at -4.25 ±0 chromosomal mu, we can speculate that it might be associated with the dumpy phenotype. Dpy-27 has been identified as an important dosage compensation gene that acts to reduce expression of both hermaphrodites X chromosomes. Similar to M77 mutant worms, dpy-27 worms exhibit shorter body phenotype than WTs. More specifically, dpy-27 is linked to sex determination in C. elegans because it encodes an ATP-binding protein, which is a homolog of the SMC4 subunit of mitotic condensing, and in conjunction with other proteins, such as MIX-1, functions as a unit to repress X-linked gene expression during hermaphrodite dosage compensation (Chuang, Albertson, & Meyer, 1994); Wormbase WS210, 2010). However, we believe that the M77 mutant gene is not associated with X-linked gene repression and sex determination; therefore, dpy-27 might not actually be the gene that causes M77 phenotype.

Since we did not find any gene that exhibited phenotypes similar to the M77 mutation gene, and at this point we do not really know whether there is a good candidate for it; it might be a gene that has not been previously described and therefore we might be the first ones to describe this gene. Moreover, due to substantial decrease in isthmus length exhibited by M77 mutants, perhaps as a result of improper signaling during morphogenesis, we believe that it might work in a similar genetic pathway like other described-known genes, such as pha-2 and pyr-1 that have been shown to cause isthmus length reduction.

Pha-2 gene encodes a homeodomain protein, which is orthologous to vertebrate Hex proteins (also known as Prh proteins), whose functions are essential for the development of B lymphocytes and organs derived from foregut endoderm (Wormbase WS210, 2010; (Mörck et al., 2004). In C. elegans, pha-2 is required for normal development and morphogenesis of the pm5 pharyngeal muscles cells, which are precursors for the bulk of the isthmus muscle cells. Mörck and colleagues showed that pha-2 mutant worms exhibit short isthmus as a result of failure of pm5 cells to properly elongate during embryogenesis. Furthermore, they showed that instead of the tight localization of adherence junctions, which form in metacorpus and posterior bulb of WT worms, pha-2 mutant worms have spread out of adherence junction molecules not only within metacorpus and posterior bulb, but also within the isthmus. Therefore, cells are not able to form tight junctions with each other, which in turn affect their ability to undergo proper isthmus elongation. Finally, pha-2 was also proposed by the researchers to be a downstream target of pha-4, which may

also function as an inhibitor of ceh-22 (an Nkx2.5 homolog) in the isthmus (Ferrier, 2009; Mörck et al., 2004).

Pyr-1, an orthologue to the human gene CPS1, which when mutated causes hyperammonemia, is another gene that exhibits shorter and thicker isthmus phenotype. Franks and colleagues (2006) have shown that pyr-1 acts upstream of proteoglycan synthesizing enzymes required for heparan sulfate proteoglycan (HSPG) synthesis and HSPGs are required for the proper pharyngeal isthmus elongation. Pyr-1 mutants are similar to pha-2 mutants in that both share shorter and thicker isthmus phenotypes compared to WTs, but they are different in that pha-2 mutants exhibit mis-positioned nuclei that were not observed in the pyr-1 mutants' isthmus (Franks, Izumikawa, Kitagawa, Sugahara, & Okkema, 2006; Mörck et al., 2004). It is clear that both, the pha-2 and pyr-1, play a critical role in the isthmus formation in C. elegans and they also give us possible reasons for the short isthmus phenotype exhibited by C. elegans in general, particularly by M77 mutants.

However, our complementation analysis data show that the M77 mutant gene can neither be pha-2 nor can it be pyr-1. Our results showed that the gene causing M77 phenotypes maps between chromosomal region -3.10 and -4.47 mu, while pha-2 and pyr-1 determined to be located on chromosome X and chromosome II between the regions of -19.53 ± 0.008 and 0.84 ±0.003 mu respectively. However, it is quite possible that the gene of interest works in a similar fashion, as pha-2 and pyr-1, in preventing the isthmus elongation.

Antibody staining of M77 worms

Myo-2::GFP and antibody staining worked for KT14, KT20, and KT36 (Figure 18). The KT14 antibody, which recognizes the basement membrane, worked as well where mutant worm (Figure 18 (A')) appeared to be missing the basement membrane in the pharynx, whereas the WT worm did not (Figure 18 (A)). These data suggest that the staining pattern observed might be either as result of malfunction in the mutant pharynx basement membrane or the antibody did not work in the mutant pharyngeal cells. At this point we do not have many worms with KT14 antibody stain to compare with and determine whether other mutant worms show the same pattern. Therefore, the KT14 antibody stain has to be repeated to see if this pattern is common amongst mutant worms. However, KT20 and KT36 that recognize the apical side of pharynx, seemed to be the same for both the WT worm and the mutant worm (Figure 18 (B, B', C, and C'). This suggests that there were no difference in antigens between the WT and Mutant worms that the KT20 and KT36 would recognize. Therefore, we have to repeat this experiment also and see if this is common amongst other worms. There were no antibody stained worms observed for KT16, MH4, and MH27 antibodies. This is primarily due to the fact that worms did not stick well to microscope slides despite trying several methods to make slides stickier.

Balancing the M77 allele

Balancing a strain is very important because it allows one to preserve a particular strain of worms with particular genetic phenotypes. Furthermore, it will make it very easy to work with the strain, for example, balanced M77 strain would allow us to know exactly which worm to pick that would be heterozygous and give us mutant worms, as opposed to transferring many individual worms and then screening for mutants, which is very time consuming and a potential way to lose a strain.

Therefore, we undertook the task to balance our M77 mutant strain as our second objective in this research project. When we carried complementation analyses to narrow down the chromosomal region for the gene casing M77 phenotype, the only strain that showed no complementation with our gene of interest was the NG2618. Since, the balancing of a strain requires that the balancer strain shows no complementation, we consequently chose the NG2618 as our balancer strain. After we carried out the balancing procedure (Figure 17), we observed that the NG2618 plates had all the characteristics we were looking for in balanced M77 strain, such as having Dpy worms and mutant worms that were not green. However, we realized that the Dpy and our mutant M77 phenotype were indistinguishable at L1 stage of development because both looked very similar, except that the Dpys would survive the L1 developmental stage, (perhaps due to their ability to eat food) while our mutant would not. We then realized that the dpy-19, which is the gene responsible for short, fat phenotype in NG2618, exhibits a phenotype very similar to our M77 mutant. Although, we were able to successfully balance the M77 mutant strain with the NG2618 deletion strain, were not able to take advantage of the balancing strain due to our inability to distinguish between the M77 mutants and Dpys at the L1 developmental stage.

Surprising/Unexpected Discovery

While examining worm plates, which were treated with 7% ethanol to produce male worms, we ended up observing quite a few number of large M77 grown-up mutant worms with normal body length but still with blunt/shortened pharynx phenotype that normally was not observed. It seems upon 7% ethanol treatment, some mutant worms that should have died as L1s, grew up to be adults (Figure 20). Therefore, it might be possible that ethanol treatment rescue the lethality of the mutant gene. It has been demonstrated by researchers that chaperones, which include the heat-shock proteins, prevent proteins from denaturing when they are under stress (Feder & Hofmann, 1999; Kern, Ackermann, Clement, Duerk, & Behl, 2010). Alcohol, which is a type of stress causing agent, can cause stress-induced protein denaturation and heat shock response. Therefore, cells turn on the heat shock proteins to cope with a particular stress, might be in turn stressed by the heat shock proteins in such a way that cause some genes, such as our M77 gene, to remain more functional when they would not be otherwise.

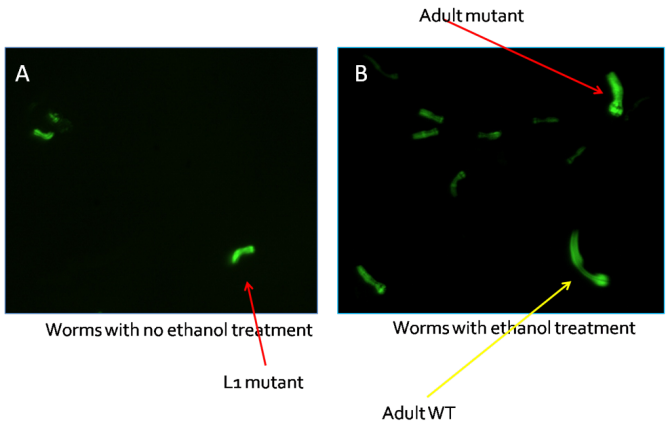

Figure 20: Worms with and without ethanol treatment: (A) Mutant worms with no ethanol exposure. (B) Mutant worms after 7% ethanol exposure.

Criticisms and Limitations

There are many aspects of this research project that can be improved in order to give our data more reliability and significance. For example, there was a procedural error in the carrying out of the interval mapping, where 1.25% gel (2.5g of agarose dissolved into 200mL of TAE or TBE buffer) was made for several experiments instead of 2.5% gel (5g of agarose dissolved into 200mL of TAE or TBE buffer). Moreover, before and after the correction of this procedural error, many lanes usually either showed no DNA at all, or were unreadable in some DNA gel electrophoreses pictures. This could have been due to a number of things such as DNA digest DraI enzyme inability to cleave, and/or denaturation of the worm DNA sample that was used to carry out the interval mapping after it was unfrozen several times from -81ºC (freezing temperature) to 20ºC to 25ºC (room temperature). Therefore, interval map for M77 mutant worms could have been carried out more carefully in order to improve the reliability of the recombination frequencies and thus prove a more accurate refinement of the interval map where our gene of interest must lie.

Although the complementation test, whereby our mutant M77 crossed with many deletion strains, produced desirable results with some deletion strains in refining the chromosomal regions to within 1.37 chromosomal map unit (between -3.1 and -4.47 interval region), more worms could have been mated with the deletion strains to improve/increase the likelihood of successful mating, particular with those in the deletion strain, such as TY1353 or MT699, in which the successful mating had never taken place. This in turn would have increased our data's significance and reliability.

In addition, the ability of maintaining and retrieving M77 or PD4792 male worms, made it very difficult to carry out the aforementioned experiments more repeatedly. Male worms were essential for this research project because they were the means of introducing our mutant allele into deletion strains by mating. Therefore, we had to use a heat-shock method, which was traditionally used by our lab to obtain male worms. However, the heat–shock method proved to be a method that would take many trials before yielding a few male worms. Even when those male worms were obtained, it was not guaranteed that they would successfully mate with the hermaphrodite to produce more male worms. In the final few weeks, we used a different method to induce male worms, and it involved treating the worms with ethanol. This seemed to be a more efficient way of obtaining male worms compared to the heat-shock method (Lyons and Hecht, 1997). However, as Hodgkin et al. demonstrated in 1979 (cited in Lints and Hall, 2009), there is an even better method that involves generating male worms in high frequency by using high incidence of males (him) mutation, which has no apparent deleterious effects on anatomy or behavior in either male or hermaphrodite sex (Lints and Hall, 2009). All in all, if we could have had a steady male population throughout this research, it would have allowed us to cross more worms with deletion worms and increase the significance of our complementation analyses data and narrow down the range of map units within which our gene of interest might lie more accurately.

Summary and Future Studies

It is vital that that the appropriate cells receive the correct signals during morphogenesis to ensure the proper organogenesis of an organism. The goal of our project was to genetically map a mutant line of C. elegans called M77 with enough precision to determine its chromosomal location and thereby determine the genetic and molecular cause of the blunt/shortened pharynx phenotype resulting from a mutation in M77 worms that might be due to a fault of morphogenesis. Our goal was also to genetically balance the M77 allele through the complementation analysis. Through complementation analyses and interval mapping, we were able to refine chromosomal region of mutant allele to chromosome III from -3 and -6 mu to -3.1 and 4.47 mu. However, within this region there is no particular known gene that exhibits our blunt/shortened phenotype. We also, were able to successfully balance the M77 mutant strain with NG2618 balancing strain; however, after balancing, we realized that it is very difficult to distinguish between our mutants and Dpy worms at L1 developmental stage, as they both exhibit a very similar phenotype. In addition, after treating L4 stage M77 hermaphrodite to obtain worms, we observed mutant worms that were well past L1 developmental stages with blunt/shortened phenotype pharynx but normal body length. This has not been observed before, and it suggested to us that we were not dealing with Dpy worms or the gene that causes a short dumpy phenotype.

At this point, we have several options for future studies. First, we can look up and find more deletion strains that cover the chromosomal region of chromosome III between -3.10 and 4.47 mu and continue further refinement of this region. Second, we can continue performing interval mapping with new sets of DNA worms to further narrow down this chromosomal region where our gene of interest must lie. Finally, we can sequence the region of -3.10 and 4.47 on chromosome III and compare it to that of normal worms to find where the irregularity lies. This will enable us to find the identity of the gene of interest more efficiently.

All of these options are good possibilities for us. One or all three of them might be carried out in the future to narrow down the chromosomal region where the mutant allele is located further to the point where we can perform transgenic rescue of the mutant worms and identity the gene of interest (Figure 21). Finally, we would like to determine

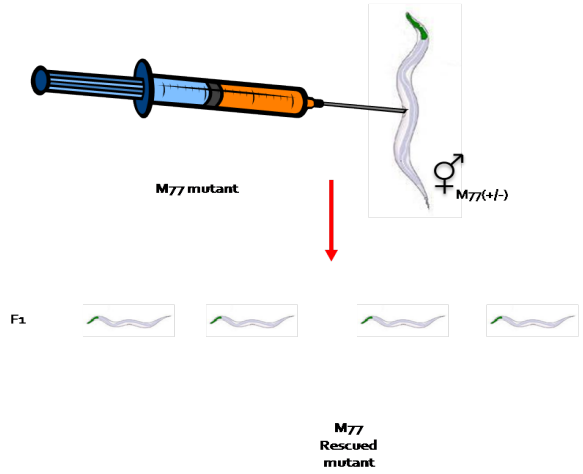

Figure 11: Transgenic rescue of the mutant worms. We will inject the WT genes into the gonads of a heterozygous hermaphrodite, where the eggs are made. Subsequently, some of the eggs that she lays will have the corrected copy of the gene, and if the animals that were mutants have the corrected copy are now not mutants, then the mutation can be said to be rescued.

the molecular pathway of M77 gene once we are able to indentify it and therefore contribute to the understanding of C. elegans genetics as well as to the overall understanding of the role of genes during development of an organism.

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