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Mutagenesis Screen in C. elegans Suggests Role of mor Genes in Pharyngeal Development

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Mutagenesis Screen in *C. elegans* Suggests Role of *mor* Genes in Pharyngeal Development

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Abstract

The study of organogenesis investigates the increasingly restrictive genetic programs that ultimately result in a single differentiated cell type. To study the genetic mechanisms orchestrating organ development, our lab chose the pharynx in *Caenorhabditis elegans* as a model system. The pharynx is a narrow tube composed of muscular epithelial tissue and is responsible for the grinding and ingestion of food in *C. elegans*. The pharynx has been so well studied that the complete lineage of cell divisions has been revealed. In addition, *C. elegans* possess a transparent body allowing researchers to track its development.

To identify the genes responsible for the specification or differentiation of muscle cells in the pharynx, our lab used the specific pharynx muscle protein myosin-2, tagged with green fluorescent protein (*myo-2*::GFP) as a visual assay. Ethyl methane sulfonate (EMS), which yields random point mutations within DNA was used to perform a mutagenesis screen of ~10,000 haploid genomes. Administration of EMS resulted in over 200 mutant lines of worms. Of these mutants, we observed anatomical variations in the pharynx that could be attributed to cell adhesion, cell fate, cell morphology, and migration in both anterior and posterior pharynx regions.

To locate the alleles responsible for disrupting *myo-2*::GFP expression in the pharynx, our lab conducted single nucleotide polymorphism (SNP) mapping (Jorgensen et al., 2005). This mapping was carried out between the wild-type *C. elegans* strain (N2 Bristol) and the genetically similar strain (CD4856 Hawaiian). To acquire accurate mapping, chromosomal and interval mapping were performed. Thus far, our lab has successfully been able to establish a linkage for 10 different mutant phenotypes to various chromosomal regions. For instance, *mor*-1, which results in a shortened, rounded pharynx, was mapped to chromosome III. Furthermore, we found another 14 similar phenotypes, which may represent at least two other genes, *mor*-2 and *mor*-3. *mor*-2 has not been cloned, but is located on chromosome IV and has been shown to yield phenotypes very similar to *mor*-1 {{81 Lewis,J.A.1977;}}. The *mor*-3 gene, a calcium/calmodulin dependent protein kinase may also have a role in abnormal pharynx development. The human orthologue of *mor*-3—dapk-1 (death-associated protein kinase 1)—is known to play a role in cell death. We believe that these *mor* genes share the same molecular pathway during development in *C. elegans* and in humans. Therefore, studying the molecular pathways of mor-1, mor-2, and mor-3 will yield a greater comprehension of muscle cell fate in the pharynx and thereby grant insight into human development.

*This author wrote the paper as a Senior thesis under the direction of Dr. Pliny Smith.

Introduction

There are very few phenomena within the experience of man more wondrous than the labyrinthine byways governing an organism's development. Most multi-cellular organisms, if not all, begin development through the fusion of an egg and a sperm to form a single-celled zygote. This single diploid cell will produce trillions of other cells, which become more specialized and ultimately create a fully functional organism. It is astonishing to think that multi-cellular organisms, such as ourselves, can emerge from the combination of the sperm and egg. In addition, it is equally astounding how cells adopt a specific cellular fate, for instance, the billions of cells that make up a heart. How does such specialization occur and how do these specialized cells come together to form specialized structures, such as organs? The focus of this thesis encompasses the development of a single cell into an organ. Specifically, our aim is to better understand the genetic mechanisms orchestrating pharyngeal development in *Caenorhabditis elegans* (*C. elegans*), a microscopic nematode.

Organogenesis: From Cells to Organs

Organs execute complex and specialized functions to sustain life. Typically, organs are composed of various tissue and cell types, which arrange in a cohesive unit fulfilling a common function. Development of organs is achieved through increasingly restrictive genetic programs, requiring temporal activation and deactivation of genes. Due to the complexity of this process, a clear understanding of the cellular and molecular phenomena driving organ development (organogenesis) has challenged researchers for decades. For a group of cells to become an organ, each undergoes cell-cell interactions, assembly into tissue, and elaborate morphogenesis, all of which are regulated at the molecular level.

Aspects of Development: Creating Cell Symmetry

Since all multi-cellular organisms originate from one cell—the fertilized egg or zygote—the problem is how the cell divides after fertilization, eventually, giving rise to diversified cell lineages rather than clones of identical cells. Usually these cells lineages arise through the differential segregation of cytoplasmic components into the different blastomeres (early embryonic cells; Wood & Edgar, 1994). These components are either already non-uniformly distributed in the unfertilized egg or are redistributed to different cytoplasmic territories of the zygote following fertilization (Wood & Edgar, 1994). As a result, the nuclei in the individual blastomeres are exposed to different cytoplasmic environments; hence, each nucleus will be subjected to factors that differentially influence gene expression. In sum, differential segregation of cytoplasmic components in blastomeres, with the ensuing activation and repression of different sets of genes in their nuclei, results in the segregation of cell lines (of groups of cells endowed with a specific developmental program).

Cell Interactions

The formation of cell boundaries during cleavage results in the isolation of specific cytoplasmic territories. It also establishes a condition whereby adjoining blastomeres can

exchange information through a communication system based on certain specializations of the cell membranes in the area of contact (Mickey, Mello, Montgomery, Fire, & Priess, 1996). The cell-cell interactions are of fundamental importance for the coordinate differentiation and specification of the individual cell lines. These labyrinthic developmental processes, which scientists try to understand, continue until all cells have acquired their fate and final position. In order to elucidate cell fate determination, researchers study simple organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster* (fly), which are amenable to experimental manipulation. Unlike the more complex organisms, mouse and chick, working with these smaller organisms, *C. elegans*, offers the chance to better understand the mechanisms orchestrating organ development.

Cellular Movement: Morphogenesis

At various times during the many forms of multicellular life, groups of cells engage in more or less extensive movements, often migrating great distances to an entirely different part of the organism (Hogeweg, 2000). For instance, the human adult cerebral cortex, which consists of six neuronal layers (I-VI), is generated from an outward migration of post-mitotic neurons that arise from mitotic progenitor neuroblasts (Tsai & Gleeson, 2005). Understanding the cellular movements is a matter of fundamental importance because cell movements are a universal feature of the embryonic development. Also, cell movements play an important role in the regeneration of lost parts and in wound closure and tissue repair. A striking example of this is seen when someone sustains a cut to arm. The laceration to the epidermal layer is repaired by the migration of neighboring keratinocytes (major type of epidermal cell; Martin & Parkhurst, 2004). In addition, some of the prominent debilitating diseases are a result of cellular movements; for example, movements of cancer cells are basic to spread of a cancer during malignancy. For these very reasons, the study of cellular movements is vital.

A Model for Organogenesis: C. elegans and the Pharynx:

Over the past decade, studies in a number of invertebrate and vertebrate model systems, including *Caenorhabditis elegans*, *Drosophila melangastor*, Xenopus, zebrafish, the chick, and the mouse, have provided insights into the genetic and cellular mechanisms regulating development (Barr, 2003; Geldziler, Kadandale, & Singson, 2004) .

In 1965, Nobel Laureate Sydney Brenner chose the microscopic free-living nematode *C. elegans* for a concentrated genetic, anatomical, and developmental investigation into the function and development of a simple nervous system (Brenner, 1974). Since then, the growth in knowledge of the biology of *C. elegans*—in terms of development, genetics, anatomy, and behavior—has led to a better biological understanding of higher organisms. For example, the widespread and naturally occurring programmed cell death (PCD) was first understood at a genetic level in *C. elegans* (Conradt & Horvitz, 1998). It was later demonstrated that these components governing *C. elegans* PCD were similar to that of humans (Metzstein, Stanfield, & Horvitz, 1998).

The biological insights gained from simple model organism has sparked an experimental and theoretical consensus that molecular mechanisms involved in development are evolutionarily conserved (Barr, 2003). For instance, the genome in *C. elegans* shares 40% homology with human DNA (Lai et al., 2000). Therefore, we would expect that *C. elegans* has many of the same genes as humans. Because of the striking genetic similarities of simple organisms and much more complicated organisms,

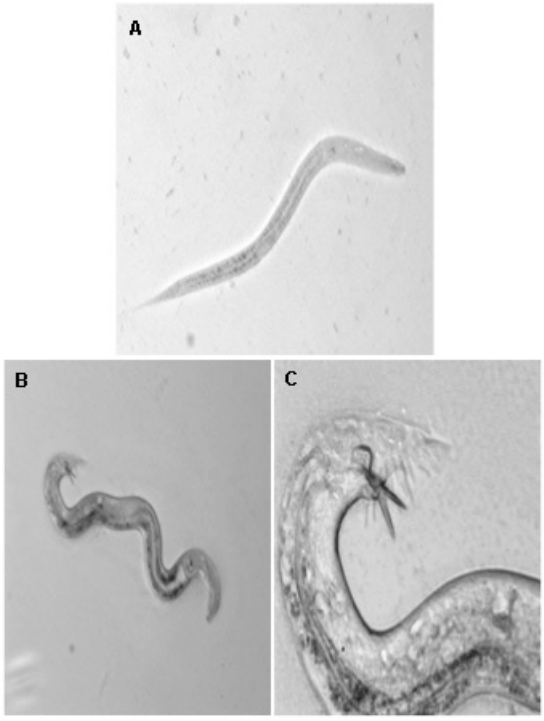

Figure 1. The *C. elegas* hermaphrodite strain and male strain. A) L2 hermaphrodite at 100x magnification (bright field). B) Adult male worm at 10x magnification (bright field). C) The male copulatory apparatus at 400x magnification (bright field).

discoveries about the organ development of *C. elegans* will provide valuable insights into the development of humans at the molecular level. For our lab to better understand the molecular complexities we have employed *C. elegans* as a model system.

C. elegans is a microscopic free-living soil nematode ~1 mm in length with myriad attractive features for the study of development. 1) *C. elegans* possesses a transparent body and an invariant cell-lineage, composed of 959 somatic cells (Sulston, Schierenberg, White, & Thomson, 1983), allowing researchers to track development at the resolution of a single-cell. 2) It has a short life-cycle, 3 days at room temperature, from egg to adult worm. In addition, it is particularly fecund, giving birth to > 300 progeny, permitting rapid genetic screens. 3) Its 97 Mb genome is completely sequenced, composing of five pairs of autosomes and one pair of sex chromosomes (Hillier et al., 2005). 4) *C. elegans* has two sexes, hermaphrodites and males, so self-fertilization of hermaphrodites or crossing with males can be manipulated to produce progeny with desired genotypes that are especially useful for genetic study (Figure 1). 5) Finally, desirable mutant strains can be preserved via freezing with liquid nitrogen. In essence, it is these traits that attract researchers to employ *C. elegans* as a model system for the study of developmental genetics.

The Alluring Pharynx

The pharynx (forgut) of *C. elegans* is an ideal organ for studying the elaborate molecular process of organ development. The pharynx functions as a rhythmic muscular organ that ingests and grinds bacteria to provide nutrition. Like most complex organs, *C. elegans* pharynx is composed of multiple cell types and exhibits complex morphogenesis. Fortunately, the anatomical position of each

of these cells has been defined via electron microscopy (Albertson & Thomson, 1976), and can be easily seen using DIC microscopy (differential interference contrast). Moreover, the advent of molecular markers, such as green fluorescent protein (GFP), has allowed researchers to track pharyngeal morphogenesis, as well as an individual cell type.

The pharynx is a narrow tube composed of muscular epithelial tissue and is responsible for the grinding and ingestion of food in *C. elegans* (Figure 2). From anterior to posterior the pharynx is divided into distinct sections: the buccal cavity, procorpus, metacorpus (anterior bulb), isthmus, and the terminal bulb. Like most intricate organs in higher organisms, the pharynx comprises of five major cell types: muscle cells (37 nuclei), nerve cells (20 nuclei), marginal cells (9 nuclei), epithelial cells (9 nuclei), and gland cells (5 nuclei; (Albertson & Thomson, 1976). Furthermore, like higher organisms, pharyngeal development is polyclonal (produced by multiple cell types; Sulston, Schierenberg, White, & Thomson, 1983), and has been shown to involve human and mammalian orthologues. For instance, the organ identity gene, *pha-4*, whose expression is necessary and sufficient for pharynx development (Mango, Lambie, & Kimble, 1994) encodes transcription factor orthologues in *Drosophila melanogaster* (FORKHEAD) and in mammals (Fox A), which are vital for gut formation (Kalb et al., 2002). Also, the transcription factor, FoxA2, orthologue of *pha-4*, is an essential component of gut development in all organisms studied to date (Carlsson & Mahlapuu, 2002). This conservation of transcription factors shared between organisms indicates that particular genetic pathways must also be evolutionarily conserved.

Other important findings suggest that the pharynx and the vertebrate heart may have descended from a common developmental process. First, like the heart, the pharynx maintains synchronous muscular contractions (Avery & Horvitz, 1989). Second, the *ceh-22* gene, encoding a NK-2 family homeodomain factor, essential for normal pharyngeal development in *C. elegans* (Okkema, Ha, Haun, Chen, & Fire, 1997), shares homology with homeobox genes, *tinman* and *nkx2.5*, which are involved in the development of cardiac muscle in vertebrates and insects, respectively. Additional evidence shows that *C. elegans ceh-22* mutants are rescued when expressing human *nkx2.5* in the muscle cells of the pharynx. This indicates that CEH-22 target gene, *myo-2* (pharyngeal muscle protein), can be activated by Nkx2.5 (Haun, Alexander, Stainier, & Okkema, 1998). These results further signify similarities between *C .elegans* and higher organisms.

Interestingly, aspects of *C. elegans* organ structure represent that of higher organisms. Specifically, the widespread tubular structures that function in digestive, respiratory, and capillary networks throughout numerous organisms also manifests in the tube-like pharynx of *C. elegans* (Portereiko & Mango, 2001). The dense interdependent networks they compose, tube structures, only transpire through intricate molecular pathways (Portereiko & Mango, 2001). One such example can be seen in *C. elegans*, where, during morphogenesis, pharyngeal primodium (ball of pharyngeal cells) reorganizes into a long narrow tube that eventually becomes a mature pharynx. Moreover, pharyngeal morphogenesis resembles aspects of tubulogenesis (tube morphogenesis), most notably kidney tubuogenesis (Portereiko & Mango, 2001). One such similarity between pharyngeal morphogenesis and kidney tubulogeneis is that both organs exhibit apical/basal polarity rearrangement (epithelial cells restructure their apical surface so the emerging pharyngeal lumen extends toward the anterior) in the epithelial cells, thus producing tubular formation (Portereiko & Mango, 2001). Together, these results give rise to a hypothesis verging on conviction that if we come to understand what drives pharyngeal development in *C. elegans,* we could learn hidden secrets of organ development in complex organisms.

Constructing the Pharynx

As mentioned earlier, *C. elegans* pharyngeal development is polyclonal (from two or more cells), commencing during the early stages of embryogenesis where restrictive genetic pathways dictate a cell's fate and future anatomic position. In order for pharynx cells—muscle cells, nerve cells, marginal cells, epithelial cells, gland cells—to acquire identity, each undergoes successive waves of gene expression governed by regulatory transcription factors. In essence, cells become more specialized with each wave of transcription activation.

Early patterning begins when the oocyte is fertilized by an amoeboid sperm, defining the posterior of the zygote (Wood & Edgar, 1994). After the first asymmetrical cleavage, a large anterior blastomere, AB cell, and small posterior blastomere, P1 cell, are produced (Figure 3). The AB cell will form the ABa and Abp blastomeres while the P1 cell forms the EMS and P2 blastomeres. At the 4-cell stage, ABa and EMS blastomeres, produce pharyngeal descendents and non-pharyngeal descendants—pharynx, neurons, epidermis, gonad, body wall muscle, midgut (Sulston et al., 1983). Interestingly, ABa and EMS blastomeres produce pharyngeal descendants via independent pathways. ABa blastomeres generate pharyngeal descendants through inter-cellular communications between blastomeres and maternally donated *glp-1* RNA (Notch receptor orthologue; Evans, Crittenden, Kodoyianni, & Kimble, 1994; Priess, Schnabel, & Schnabel, 1987; Gene Function and Phenotype Table I). On the other hand, the EMS blastomere produces pharyngeal cells through interactions with the maternally supplied genes, *skn-1* and *pop-1* (Bowerman, Eaton, & Priess, 1992; Lin, Thompson, & Priess, 1995). Ultimately, these two lineages, ABa and EMS, direct the formation of the pharynx.

ABa Lineage: The Anterior Pharynx

Typically, the pharynx is produced through two particular cell lineages: ABa and EMS. At the four-cell stage of development and forward, these two cell lineages, ABa and EMS, are responsible for the generation of both pharyngeal and non-pharyngeal cells (Sulston, Schierenberg, White, & Thomson, 1983). AB descendants, ABa and ABp, initially have the ability to become ectodermal cell types, such as skin and nerve cells (Good et al., 2004). It is through inter-cellular signaling (GLP-1/Notch) that these early blastomeres are ascribed their fate. The first Notch signaling occurs between blastomeres P2 (P1 descendant) and ABp (AB descendant), whereby P2 cells induce ABp descendants to retain their ectodermal cell fates (Good et al., 2004). This induction occurs when APX-1, a transmembrane and secreted protein, binds GLP-1 for an inductive interaction that specifies the fate of the ABp blastomere. The anterior daughter cell, ABa, which is responsible for the anterior half of the pharynx, does not come in contact with P2, therefore retaining its primary fate (Good et al., 2004). However, at the 12-15 cell stage the activation of *lag-1* transcription factor by an unknown GLP-1/Notch ligand via EMS descendant, MS, induces PHA-4 (organ identifier protein) and REF-1 (bHLH transcription factor) expression (Neves & Priess, 2005; Smith & Mango, 2007). This eventually leads to anterior pharynx formation. In brief, the formation of the anterior pharynx, is not, in fact, a default for ABa, but relies on a subsequent positive inductive interaction.

In addition to signaling ABa descendants, MS itself is a mesodermal precursor responsible for the development of the posterior pharynx. If MS is killed prior to the second

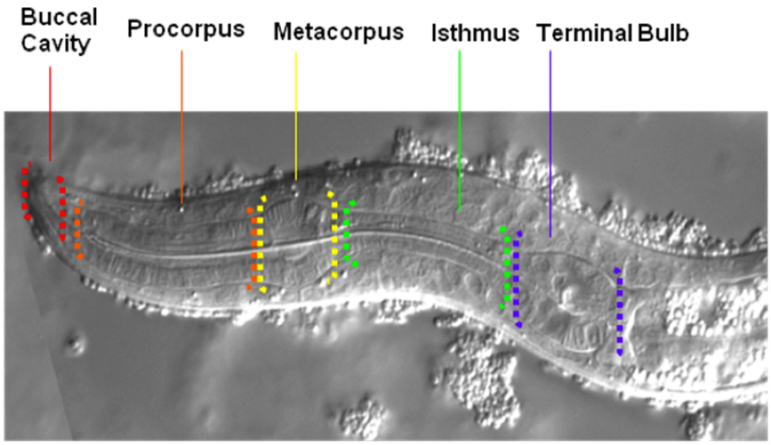

Figure 2: Anatomical subgroups of *C.elegans* pharynx: red brakets, Buccal cavity; orange brackets, Procorpus; yellow brackets, Metacorpus; green brackets, Isthmus; purple brackets, Terminal bulb.

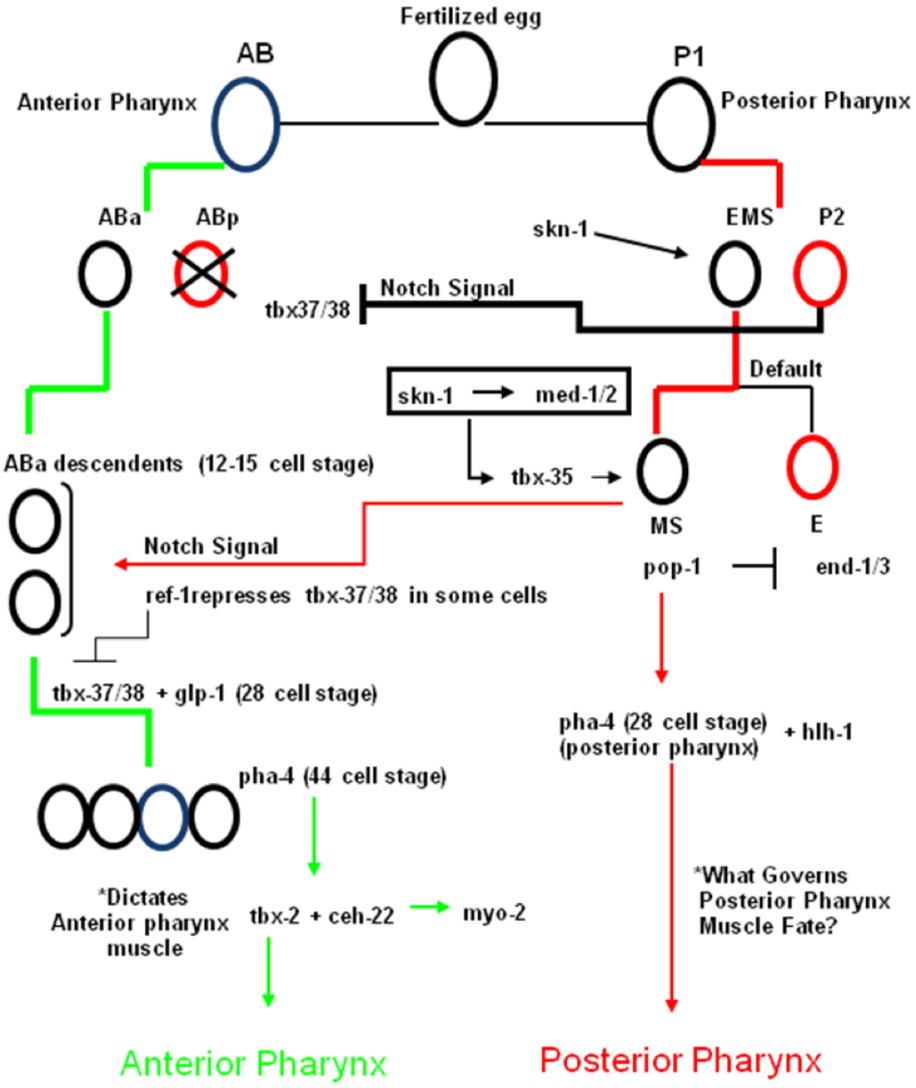

Figure 3. AB all of its early descendents express receptors GLP-1/Notch or LIN12/Notch…..The First Notch Interaction occurs at the 4 cell stage when the posterior daughter of AB, called Abp, contacts P2 that expresses a notch ligand, thus causing the Abp to take on a new fate, ectodermal precursors.

Notch signaling (12 cell stage), no pharyngeal cells are produced (Hutter & Schnabel, 1994). On the contrary, if MS descendants are killed after the second Notch signaling, no posterior half-pharynx cells develop, but the anterior half-pharynx cells do form (Good et al., 2004).

After these early inductive interactions are complete, the developing embryo is on the verge of even greater specialization. For instance, at the 24 cell stage, 8 ABa descendents express two redundant proteins, T-box transcription factors, TBX-37 and TBX-38 (*tbx*-37/38), whose expression is restricted to these cells by REF-1 in order to achieve proper anterior development (Good et al., 2004; Neves & Priess, 2005). The expression of TBX-37/38, works in conjunction with Notch signaling to induce PHA-4, which activates competent anterior pharynx cells. Neither Notch signal transduction nor TBX37/38 alone are adequate for mesodermal induction (Good et al., 2004). Moreover, although MS signaling is essential for ABa descendants to become mesodermal precursors that form the anterior pharynx, it is the primary Notch signaling (P2, four cell stage) that inhibits cells from becoming mesodermal precursors via TBX37/38 repression (Good et al., 2004). The absence of the first Notch signaling leads to the hyperinduction of pharyngeal tissue, further exemplifying the importance of GLP signaling (Hutter & Schnabel, 1994). In short, TBX-37/38 activity is repressed and activated by GLP-1. For this reason, a double requirement for GLP-1 exists, first as a receptor mediating the repression of pharynx formation by the action of P1 and ABp, and then as the receptor mediating positive induction of pharynx formation through the action of MS on ABa descendants.

At the 44 cell stage, *pha-4,* pharyngeal identity gene that activates different genes at different times, expression occurs through a combination of TBX-37/38 and Notch signaling (Good et al., 2004). *pha-4* loss-of-function mutants don't develop a pharynx (Mango et al., 1994). Yet, ectopic *pha-4* expression yields extra pharyngeal cells (Arnone, 2002). It is clear that *pha-4* specifies pharyngeal identity in ABa and EMS descendents. Like *pha-4*, *tbx-37*/*38* mutant embryos are unable to form an anterior pharynx (Aph phenotype). As shown, PHA-4 expression isn't initiated by the ABa descendents of *tbx-37*/*38* mutant embryos (Good et al., 2004). *tbx37/38* mutant embryos appear to express PHA-4 in intestinal and rectal cells, however, most pharynx cells appear to be missing (Good et al., 2004). These results indicate that PHA-4 is essential for pharyngeal development; yet, they do not explain how pharyngeal cell fates are acquired, specifically muscle cell fate, nor did they (the results) explain factors regulating muscle activity.

One of the first links to pharyngeal muscle was the myogenic gene, *ceh-22*. The *ceh-22* gene, which is expressed in pharyngeal muscle, induces muscle activity by targeting the myosin heavy chain gene, *myo-2* (Okkema & Fire, 1994). The loss of *ceh-22* results in feeding abnormalities due to improper muscle function (Okkema & Fire, 1994). Yet, the presence of muscle cells in *ceh-22* mutants reveals that some other factors govern pharynx muscle fate. Research by (Smith & Mango, 2007) shows that *tbx-2* is another key component for pharyngeal muscle fate of the anterior pharynx. Inhibiting the function of *tbx-2* arrests the development of anterior pharynx muscle, however posterior pharynx remains unchanged.

In sum, the development of the anterior pharynx is attained through the ABa lineage as well as inter-cellular communications with the neighboring lineage, EMS. One would think that losing a protein involved in the muscle fate of an organ would result in complete loss of muscle for that organ. Remarkably, this is not the case in the pharynx of *C. elegans*. When the transcription factor, TBX-2, is inhibited, the worm develops normally except for the lack of just anterior pharynx muscle. Accordingly, this prompts the question, what other factors are contributing to posterior pharyngeal muscle fate?

EMS Lineage: The Posterior Pharynx

Unlike the ABa lineage, which relies on early inter-cellular *glp-1* activity, the EMS lineage requires signaling from two maternal genes in order to generate pharyngeal cells, namely *skn-1* (bZIP-related transcription factor) and *pop-1* (Bowerman et al., 1992; Lin et al., 1995). Specifically, the ABa linage is guided by inter-cellular interactions (*glp*-1) and the EMS lineage is achieved by a default mechanism (Figure 3). *skn-1* participates in the development of the EMS blastomere at the 4-8 cell stage, whereby it activate*s med-1* and *med-2* (*med*-1/2), hence promoting specification of MS blastomere (Bowerman et al., 1992; Broitman-Maduro, Lin, Hung, & Maduro, 2006). The absence of *skn-1* results in no pharynx, because the EMS descendents develop into C blastomere rather than MS blastomere. In contrast to its cousin, MS blastomere, the C blastomere does not apply Notch ligands (*glp*-1) to signal to the ABa blastomeres. Unlike the *skn-1* (-) embryos, which lack the pharynx altogether, *med-1,2* (-) embryos manifest AB-derived anterior pharynx (Maduro, Meneghini, Bowerman, Broitman-Maduro, & Rothman, 2001). Another critical element of MS specification is *pop-1. C. elegans pop-1* (-) embryos reveal a MS to E transformation due POP-1's inability to repress *end-1/3* (Lin et al., 1995). It is thought that MS fate is attributed to *med-1/2* by targeting the transcription factor *tbx-35*, which in turn activate *pha-4*.

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