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ANALYSIS OF *C. ELEGANS* VULVAL MORPHOGENESIS REVEALS NOVEL MECHANISMS FOR INITIATION AND MAINTENANCE OF BIOLOGICAL TUBES

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ABSTRACT

The formation of biological tubes is common to the development of all metazoans. A simple model for studying all aspects of this complicated developmental process is the *C. elegans* vulva. This simple epithelial tube consists of seven toroidal-shaped cells surrounding a central lumen. Using this model, I have uncovered novel mechanisms in lumen initiation and lumen maintenance during vulval tubulogenesis. I have shown that the putative palmitoyltransferase EGL-26, belonging to the NlpC/P60 family of enzymes, is specifically required late in dorsal vulval morphogenesis. Moreover, I have shown that mutation of *egl-26* results in a collapse of the dorsal vulval lumen. I also show that the conserved catalytic residues of EGL-26 are required for function *in vivo*, providing the first example of a function for this family of proteins in metazoan development. In addition, I have closely analyzed the role of the gonadal anchor cell in vulval morphogenesis. Using several mutants, I have shown that invasion of the vulva epithelium by the anchor cell is required for initiation of the dorsal vulval lumen. In cases where the anchor cell does not invade properly, no dorsal lumen is formed. I have confirmed that this effect is specific to morphogenesis and not due to fate or polarity defects. This work leads to a model of vulval tubulogenesis that requires the anchor cell to initiate dorsal lumen formation and EGL-26 function to maintain the lumenal structure, suggesting that initiation and maintenance are controlled separately. An understanding of these mechanisms in the *C. elegans* vulva will likely lead to insight into potentially conserved mechanisms in higher eukaryotes.

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PREFACE

This dissertation contains work which has been published and is in press. The majority of Chapter 2 has been published in Developmental Biology.

Estes, K. A., Kalamegham, R. and Hanna-Rose, W. (2007). Membrane localization of the NlpC/P60 family protein EGL-26 correlates with regulation of vulval cell morphogenesis in Caenorhabditis elegans. *Developmental Biology* **308**, 196-205.

Rasika Kalamegham is responsible for the anti-PAR-6 antibody staining of the vulva, as well as some of the initial cloning involved in the protein localization experiments using the CAAX motif.

The majority of Chapter 3 is in press in Developmental Biology. Genevieve Streb assisted in sequencing the *unc-6* locus in *ku243* mutants.

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Chapter 1

Introduction

Tubulogenesis

Forming tubes is a widespread occurrence in the development of all metazoans. Most of the organ systems in any animal are formed of tubes or a series of connected tubes. The process of creating a biological tube is complicated and involves dynamic cell shape changes, migrations, interactions between cells and often between different tissues, and all of this must occur in a spatially and temporally controlled manner to ensure proper development. In humans, when these tubes are not formed or connected properly, the course of treatment is often surgery as the underlying genetic and molecular causes are still largely unknown. An understanding of such a common developmental event as tubulogenesis is necessary for our understanding of metazoan developmental biology.

Biological tubes vary in shape, size, and function, and therefore, formation of these structures is expected to be diverse. However, many aspects of tubulogenesis are common to all types of tubes studied thus far. First, all tubes are polarized, meaning that all the cells have an apical membrane facing the lumen of the tube. However, tubes can form from tissue that is previously polarized, as is the case with the *C. elegans* vulva, or polarization can occur during the process of tubulogenesis, as is the case in mammary

gland development (Hogg et al., 1983; Yohki Hieda, 1996). Polarization is important for many reasons; if polarization is perturbed, morphogenesis is abnormal and the proper tubular structure is not formed (Yeaman et al., 1999). Once the tube is formed, the function of the tube, including proper trafficking of nutrients, proteins, or other components to and from the lumen and adjacent tissues relies on the polarized nature of the cells.

Another obvious component of a biological tube is the central lumen. The final structure of the lumen and its content vary greatly. Some tubes have very elaborate branching structures. Other, simpler tubes are composed of one single cell or one multi-cellular tubular structure surrounding a central lumen. Interestingly, lumen creation does not differ as dramatically as the diversity of lumens would suggest (reviewed in (Hogan and Kolodziej, 2002). The basic problem of lumen formation is creating a space between cells which are connected. Some lumens are formed within a single cell, like in the excretory canal in *C. elegans* (Buechner et al., 1999), as well as certain parts of the Drosophila trachea (Uv et al., 2003). Some lumens are formed by cells separating from each other, creating a space between them as seen in zebrafish vascular development (Jin et al., 2005). Other lumens are formed via apoptosis of cells that occupy the future lumenal space as in formation of the proamniotic cavity during mouse embryogenesis, and morphogenesis of the mouse submandibular gland (Coucouvanis and Martin, 1995; Tina Jaskoll, 1999). Another very common mechanism of lumen formation is epithelial invagination, as seen in the *C. elegans* ventral vulva (Sharma-Kishore et al., 1999). Sometimes varying types of lumen formation are observed in a single organ, as in the *Drosophila* trachea (Uv et al., 2003). Despite all of the *in vivo* studies, as well as studies

in 3D culture systems, there are still many questions regarding lumen formation.

Investigation of these very basic processes of tubulogenesis is difficult in most metazoans because of the sheer number of cells and tissues involved. The ability to dissect something as basic as lumen formation becomes complicated when it occurs during periods of development when an animal is in utero or when mutation of a gene resulting in the desired phenotype also causes lethality. Using a simple model system to study these processes is a powerful way to characterize tubulogenesis down to the level of the behavior of a single cell. In my investigations of tubulogenesis, I use the *C. elegans* vulva, a tubular organ consisting of seven toroidal-shaped cells. The well-characterized behavior of these cells during development makes it an excellent model system for discovering mechanisms involved in tubulogenesis. And given the evidence that mechanisms employed during tubulogenesis seem to be at least partially conserved across different species, the vulva will likely provide insight into mechanisms that may have previously been unnoticed in larger, more complex systems.

Caenorhabditis elegans **as a model system**

Caenorhabditis elegans is a free-living soil nematode approximately 1 mm in length. Established by Sydney Brenner as a model organism in the 1970s, *C. elegans* has been used to study an ever-growing range of topics from basic cell biology to the complexities of neurobiology and aging. Many features of this small worm make it an appealing model system. *C. elegans* are easily cultured in a laboratory setting, have a short generation time, is the first metazoan to have its genome sequenced, and has an

invariant cell lineage that has been mapped from embryo to adult, allowing one to study development at the cellular level (Brenner, 1974; Sulston and Horvitz, 1977; The *C.elegans* Sequencing Consortium, 1998). *C. elegans* has two sexes, hermaphrodite and male, thus providing an excellent genetic system, allowing one to obtain an exact genetic copy via self-fertilization, as well as the ability to cross different genotypes by mating (Brenner, 1974). In addition, there are many easily-identifiable phenotypes, making genetic screens a useful tool to identify new genes.

C. elegans body plan is very simple, consisting primarily of intestine and gonad. The gonad is a U-shaped organ, with two arms extending away from the centrally-located uterus. The distal arms contain a syncytium of mitotically active nuclei that travel toward the proximal ends and go through several phases of maturation before eventually forming distinct oocytes (reviewed in Lints and Hall, 2002). Mature oocytes are fertilized in the body of the hermaphrodite in the spermatheca, a specialized organ that stores sperm, which is located at the proximal end of each gonad arm adjacent to the uterus (Hirsh et al., 1976). The oocytes are fertilized either by the sperm of the hermaphrodite, or sperm from a male that has entered the vulva via mating and traveled to the spermatheca. Once fertilized, the zygote passes through the uterus and develops for a time before passing through the vulva to the outside environment. Several hours later, the animal hatches in the first of four larval stages before entering adulthood. These stages, called larval stages L1-4, occur over approximately 2 days post-hatching (Sulston and Horvitz, 1977; Sulston et al., 1983; Sulston and White, 1980). Each larval stage is separated by a molting event in which the cuticle of the animal is shed (Cassada and Russell, 1975). The development of many different organs occurs throughout the larval stages, including the vulva, which

is detailed below. Once *C. elegans* reaches the adult stage, approximately 45-50 hours post hatching, they are able to mate, fertilize and lay eggs (Sulston and Horvitz, 1977). Eggs are laid for 3-4 days and 300+ progeny are produced by self-fertilization in a single wild-type hermaphrodite.

Vulva development

The *C. elegans* vulva is an elegant model used to study cell fate determination, cell signaling, cell fusion, morphogenesis and organogenesis (reviewed in Sternberg, 2005). This simple tubular organ consisting of seven multi-nucleate cells functions as the connection between the uterus and the outside environment facilitating egg-laying and mating. It is non-essential, making it ideal to study perturbations in its development. Animals that lack a vulva or a proper connection between the uterus and vulva develop into what is called a 'bag of worms' where the self-progeny are fertilized within the body of the worm but cannot be laid. Instead they continue to develop and hatch inside the mother, resulting in maternal death. They eventually escape to the outside environment by eating their way through the maternal body wall. This phenotype is very easily identifiable.

Development of the vulva begins in L1 with the birth of the vulva precursor cells (VPCs) (Sulston and Horvitz, 1977). These hypodermal cells are arranged in a line along the ventral side of the animal. They are named P3.p-P8.p, and all six are competent to develop into the vulva (Kimble, 1981; Sternberg and Horvitz, 1986; Sulston and White, 1980). Vulval induction begins when an epidermal growth factor (EGF)-like signal,

called *lin-3*, is secreted from a uterine cell called the anchor cell (AC) which lies directly above the VPCs (Hill and Sternberg, 1992) (Fig **1-1**). In wild type animals, the three central VPCs become the vulva, P5.p-P7.p (Fig **1-1**). P6.p, the central cell, receives the highest concentration of *lin-3* signal from the AC and forms the primary cell lineage. P5.p and P7.p are specified via the Notch and Wnt pathways and adopt the secondary cell fate (Chen and Greenwald, 2004; Green et al., 2008; Greenwald et al., 1983). Interestingly, the Notch pathway is activated in P6.p upon receiving the *lin-3* signal, and P6.p subsequently sends a lateral inhibitory signal to P5.p and P7.p causing them to adopt a secondary cell fate (Chen and Greenwald, 2004; Greenwald et al., 1983; Sternberg, 1988; Yoo et al., 2004). P3.p, P4.p, and P8.p fuse to the surrounding hypodermal cell in wild type animals, however, they are competent to form vulva cells and do so in various mutant backgrounds (Kimble, 1981; Sternberg and Horvitz, 1986; Sulston and White, 1980).

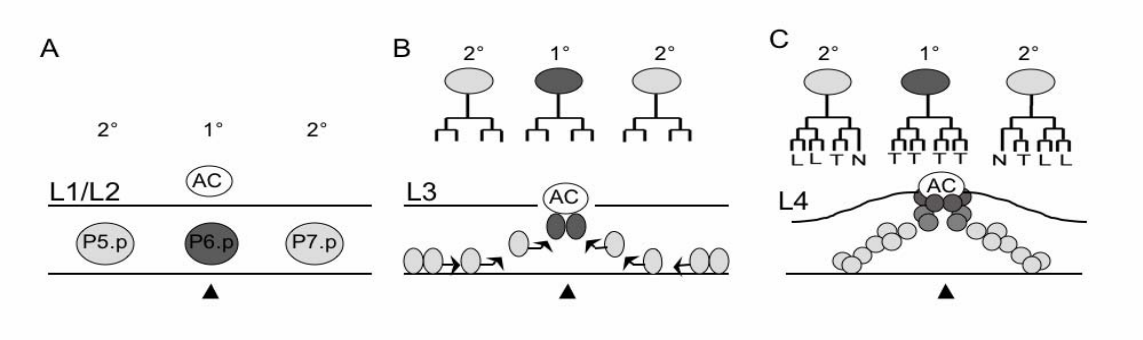

Figure **1-1**: Schematic of vulva development. 1° cells (vulE and vulF) indicated in dark grey, 2° cells indicated in light grey. (A) L1/L2 stage when P5.p, P6.p and P7.p are first specified by the AC to adopt a vulval cell fate. (B) L3 stage. Top panel: lineage of 1° and 2° cells. Bottom panel: The AC sits above vulva in the uterus before invasion. Midline-directed migration of 2° cells (indicated by arrows) under the 1° cells drives invagination and creates the ventral lumen space (arrowhead indicates midline). (C) L4 stage. The AC has invaded the basement membranes and resides between vulF cells where a small lumen is being formed. 2° cells already surround a large ventral lumen.

B) L3

Once the VPCs receive the inductive *lin-3* signal from the AC, they begin to divide into twenty-two cells. As these divisions occur, the cells also begin to invaginate dorsally toward the AC and uterus and away from the cuticle (Sharma-Kishore et al., 1999). This process seems to be driven by cells of the secondary lineage migrating toward the vulval midline, and forcing the primary lineage to move dorsally toward the uterus (Fig **1-1B**). Once the cells have finished their divisions, fusions between cells which will form each of the seven toroids begin (Sharma-Kishore et al., 1999). Descendents of P5.p and P7.p send out processes toward each other to fuse with their

counterpart on the opposite side of the vulval lumen while descendents of P6.p are born in proximity to each other and fuse with each other (Dalpe et al., 2005; Sharma-Kishore et al., 1999). The toroids are labeled vulA-vulF starting at the ventral side (Fig **1-2B**). The secondary lineage, descendents of P5.p and p7.P form vulA-vulD, and the primary lineage, descendents of P6.p, form vulE and vulF. vulE and vulF constitute the dorsal vulva, and vulF forms the functional connection with the uterus by contacting the uterine cells directly (Sharma-Kishore et al., 1999). This connection is essential for a functional egg-laying apparatus.

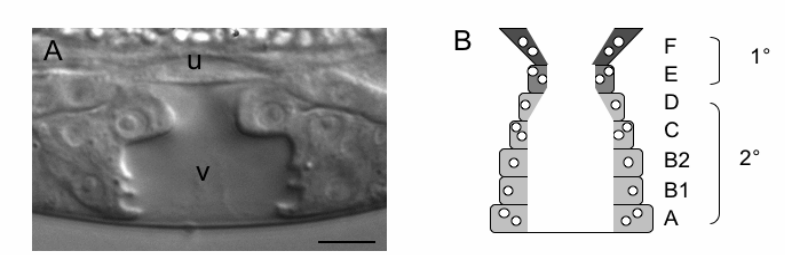

Figure **1-2**: DIC photomicrograph and schematic of mid-L4 vulva. (A) DIC image of wild type mid-L4 vulva. v indicates vulval lumen, u the uterus. (B) Cross-section of the final toroid structure of the vulva. 1° cells indicated in dark grey, 2° cells indicated in light grey. Vulva toroids are indicated, and number of nuclei per toroid is indicated with white dots. Scale bar: 5μm.

egl-26

As mentioned above, *C. elegans* is an excellent model for genetic studies, particularly in identifying genes important for egg-laying. The *egl-26* gene was first identified in a screen for mutants with Egg-laying (Egl) defects (Trent et al., 1983). New alleles were found in a screen for Egl mutants associated with specific vulval morphology defects (Hanna-Rose and Han, 2002). In *egl-26* mutants, the most dorsal vulval cell, vulF, adopts a "closed" morphology with no apparent inner lumen as opposed to the expected toroidal morphology (Fig **1-3B**). This results in a blockage between the vulval and uterine lumens, a Connection of gonad (Cog) defect. There are three characterized alleles of *egl-26* (Table **1-1**). None of the alleles are completely penetrant for the Egl phenotype.

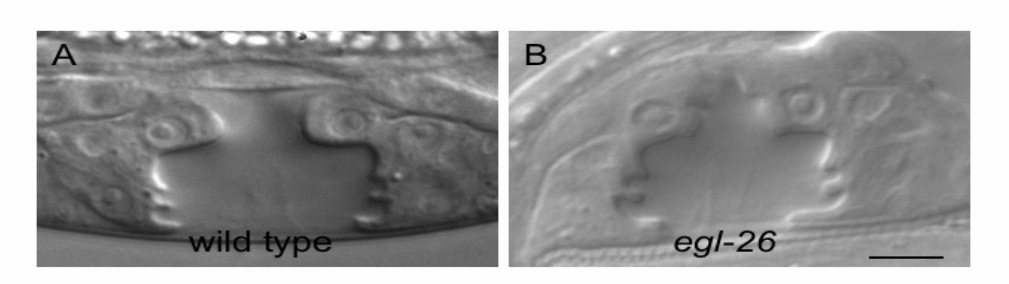

Figure **1-3**: DIC image of wild type *egl-26* mutant vulval morphology at mid-L4 stage. (A) wild type vulva with wide open vulF lumen. (B) *egl-26* mutant vulva with occluded vulF toroid. Scale bar: 5μm.

Table **1-1**: Penetrance of Egl phenotype in *egl-26* mutants

alleles	**% Egl**	**n**
ku228	83	175
ku211	75	92
n481	55	53

Adapted from (Hanna-Rose and Han, 2002)

Initial studies of *egl-26* by Hanna-Rose and Han demonstrated that EGL-26 expression in the primary vulval lineage, descendents of P6.p, rescues the vulval morphology defect. In addition, a translational GFP fusion that rescues the mutant phenotype showed expression in the vulva, the cells of the spermatheca, around the mouth, and lining the rectum and the pharynx (Hanna-Rose and Han, 2002). Interestingly, expression was not observed in vulF, the cell that exhibits the morphology defect. Taken together with the evidence that EGL-26 expression in the primary vulval lineage rescues the mutant phenotype, EGL-26::GFP expression suggests that expression in vulE is required for EGL-26 to function in vulval morphogenesis. This suggests *egl-26* acts non-cell autonomously (Hanna-Rose and Han, 2002). Additionally, EGL-26::GFP has a distinctly apical membrane localization despite the absence of an obvious predicted alpha-helical membrane-spanning domain (Hanna-Rose and Han, 2002).

NlpC/P60 Superfamily of Enzymes

EGL-26 shares sequence motifs with the NlpC/P60 superfamily of enzymes (Anantharaman and Aravind, 2003). This family includes proteins that range in

biochemical function from cell wall peptidases in prokaryotes to palmitoyltransferases in mammals (Anantharaman and Aravind, 2003). The defining motifs of this family are the H-box domain and the NC domain (Fig **1-4**) (Anantharaman and Aravind, 2003; Hughes and Stanway, 2000). The H-box contains a conserved histidine, and the NC domain contains a conserved cysteine (Hughes and Stanway, 2000). These residues are essential for catalytic function of this family of enzymes (Anantharaman and Aravind, 2003; Xue et al., 2004). Interestingly, all three *egl-26* alleles isolated in EMS screens are mutations in residues residing within these catalytic motifs, but not the putative catalytic residue itself (Fig **1-4**).

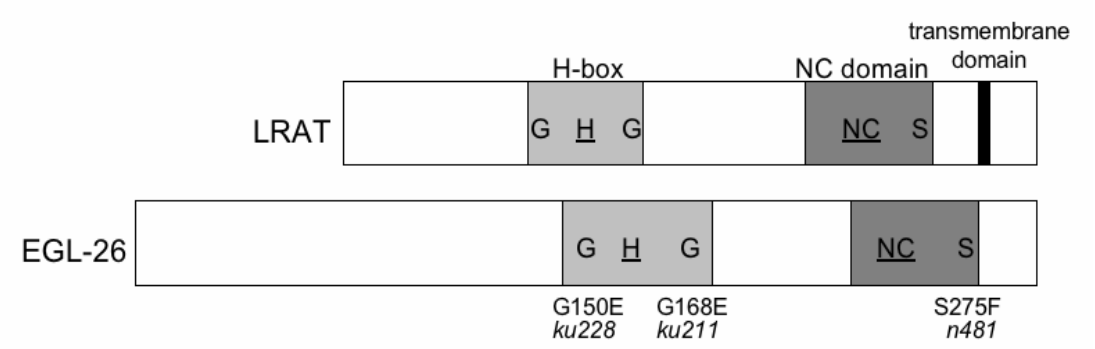

Figure **1-4**: Schematic comparing protein motifs between EGL-26 and LRAT. The positions of the conserved H-box (light grey boxes) and NC domains (dark grey boxes) that characterize eukaryotic NlpC/P60 superfamily proteins are indicated. The defining histidine (H) and asparagine/cysteine (NC) are underlined in the H-box and NC domain, respectively. The thick black line represents the LRAT transmembrane domain. EMS induced alleles of *egl-26* (*ku228*, *ku211* and *n481*) and the substitution mutations that they cause are indicated beneath the conserved amino acids that they alter. Sequence identity/similarity between EGL-26 and LRAT is 34/69% in the H-box and is 38/59% in the NC domain.

EGL-26 belongs to a subfamily that includes the mammalian proteins LRAT (lecithin retinol acyltransferase) and Hrasls3 (HRAS-like suppressor 3) as well as the picornovirus 2A proteins (Anantharaman and Aravind, 2003; Hughes and Stanway, 2000). The mammalian protein most closely related to EGL-26 is LRAT, which is a biochemically well-characterized palmitoyltransferase (Fig **1-4)** (Anantharaman and Aravind, 2003; Rando, 2002; Xue et al., 2004). LRAT palmitoylates all-*trans*-retinol (vitamin A), producing all-*trans*-retinyl esters necessary for rhodopsin chromophore production in the retinal pigment epithelium (Barry et al., 1989; MacDonald and Ong, 1988; Saari and Bredberg, 1989; Shi et al., 1993).

Enzymes in the NlpC/P60 superfamily are frequently associated with or function at the membrane (Anantharaman and Aravind, 2003; Hughes and Stanway, 2000). For example, a predicted C-terminal transmembrane domain of LRAT spans the membrane, targets the protein to the ER and is required for acyltransferase function in COS-7 cells (Moise et al., 2007). This is an interesting observation given that EGL-26::GFP expression is observed at the apical membrane despite the absence of a predicated transmembrane domain (Hanna-Rose and Han, 2002).

I have studied the role of *egl-26* in vulval tubulogenesis using several approaches. First, I further characterize the morphology defect in *egl-26* mutants. EGL-26 seems to play a role specifically in morphogenesis of the dorsal vulval toroid, vulF. Hanna-Rose and Han suggested the lumen in vulF was absent. I present evidence that mutation in *egl-26* causes a collapse of the dorsal vulval lumen that is initially established. I also examine EGL-26 protein function *in vivo*. To confirm that EGL-26 is indeed a member of the NlpC/P60 family, I have shown that the conserved catalytic residues are important

for function *in vivo*. I also present evidence that sub-cellular localization plays a role in protein function. This is the first evidence demonstrating a role for this family of proteins in metazoan development and may give insight into the function of family members that are currently unknown.

Dorsal vulval lumen formation

My analysis of *egl-26* provides insight into vulva-specific effects on dorsal vulval morphogenesis. However, other important cellular interactions also occur to ensure a proper connection between the uterine and vulval lumens. During vulval morphogenesis, the uterine AC is actively involved in specifying vulval cells and comes into direct physical contact with the vulva. Beginning in L3, the AC signals via *lin-3* to the VPCs to adopt a vulval cell fate. A few hours later, the AC invades between the most dorsal vulval cells and resides for a time in what will become the lumen of the vulF toroid (Fig **1-1**) (Kimble and Hirsh, 1979; Newman et al., 1996; Sherwood and Sternberg, 2003). At this time, the AC also helps differentiate vulF cell fate from that of vulE within the primary lineage (Wang and Sternberg, 2000). As vulval morphogenesis

continues, the AC retreats into the uterus via fusion with a uterine cell called the uterine seam cell, leaving an open lumen in vulF to connect with the uterine lumen (Newman et al., 1996). Previous studies indicate that failure of the AC to retreat can impair vulval function (Cinar et al., 2003; Hanna-Rose and Han, 1999). However, the functions of the initial invasion event as well as the consequences of mis-positioning of the AC within the vulva on vulval morphogenesis have not been investigated.

Successful AC invasion requires two major events. The first is guidance toward the vulva. UNC-6 (Netrin) and one of its two receptors, UNC-40 (DCC/Neogenin), are required to guide AC migration toward the vulva (Asakura et al., 2007; Hedgecock et al., 1990; Ishii et al., 1992). UNC-6 is implicated in many axon and cell migration events (Hedgecock et al., 1990). It is a secreted molecule that can induce an attractive or repulsive response depending on the receptor expressed in the signal-receiving cell (Merz et al., 2001). There are two known UNC-6 receptors in *C. elegans*, UNC-40 (DCC/Neogenin) and UNC-5 (Hedgecock et al., 1990). While both of these receptors are responsible for a repulsive response to UNC-6 when expressed together, UNC-40 guides cells toward an UNC-6 signal (Merz et al., 2001). UNC-40 is the only receptor that plays a role in AC migration (data not shown, (Hedgecock et al., 1990), and thus the only receptor used in this study. *unc-6* and *unc-40* mutants display a variety of phenotypes, including Uncoordinated (Unc) and Egl. In addition, the AC is often found mis-positioned within the uterus and never or incompletely invades the vulva (Hedgecock et al., 1990). UNC-6 and UNC-40 function in AC migration is reported to cause the Egl defect (Hedgecock et al., 1990). The second major event required for proper AC invasion of the vulva is the breakdown of the basement membranes that reside between

the uterus and the vulva by the AC (Sherwood and Sternberg, 2003). *fos-1* is a transcription factor that is required cell-autonomously for the AC to break down these basement membranes, but does not play a role in targeting the AC toward the vulva (Sherwood et al., 2005). In *fos-1* mutants, the AC remains in the uterus and does not invade the vulva (Sherwood et al., 2005).

I have observed a previously un-characterized vulva morphology defect, that upon initial observation, very closely resembled the dorsal vulval morphology defect seen in *egl-26* mutants. *unc-6*, *unc-40* and *fos-1* mutants all exhibit this morphology defect. I have identified that the cause of this morphology defect is lack of or incomplete AC invasion and penetration of the vulva. Fate specification and polarity are normal in the vulval cells, suggesting that like EGL-26 function, AC invasion of the vulva is required specifically for morphogenesis. I also present evidence that no dorsal vulval lumen is present in these mutants, suggesting that AC invasion is required for dorsal vulval lumen initiation. These observations suggest that ventral and dorsal lumenal development are separate events, and that dorsal lumenal initiation is distinct from lumenal maintenance. My work suggests a model for *C. elegans* vulval tubulogenesis that involves initiation of the dorsal lumenal structure by AC invasion, followed by function of EGL-26 to maintain the dorsal lumen and create a functional uterine-vulval connection.

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Chapter 2

Membrane localization of the NlpC/P60 family protein EGL-26 correlates with regulation of vulval cell morphogenesis in *C. elegans*

Introduction

Vulval morphogenesis in *C. elegans* generates a stack of toroidal cells enclosing a tubular lumen. As introduced in Chapter 1, mutation of *egl-26* is associated with malformation of vulF, the most dorsal toroid in the stack, resulting in a blocked lumen and an egg-laying defect (Hanna-Rose and Han, 2002). To more closely examine this novel mutant phenotype, I have further characterized the vulval morphology defect. I will present evidence that vulF retains the expected gene expression pattern, functions in signaling to the uterus and retains proper polarity when *egl-26* is mutated, all suggesting that mutation of *egl-26* specifically results in aberrant morphogenesis as opposed to abnormal fate specification. Recent computational analysis indicates that EGL-26, which was previously characterized as novel, belongs to the LRAT (lecithin retinol acyltransferase) subfamily of the NlpC/P60 superfamily of catalytic proteins (Anantharaman and Aravind, 2003). To examine whether the catalytic residues of this family that are conserved in EGL-26 are functional, I use site-directed mutagenesis and demonstrate that they are indeed required *in vivo*. I also demonstrate that the conserved residue serine 275 is important for the apical membrane association and function of EGL-

26. Finally, additional mutagenesis of this residue suggests that EGL-26 attains its membrane localization via a mechanism distinct from that of LRAT.

Results

Developmental role of EGL-26

A specific vulF morphology defect is associated with all EMS-induced alleles of *egl-26* (Hanna-Rose and Han, 2002). However, none of the EMS induced alleles are a predicted molecular null nor are they fully penetrant for the morphology or Egl defects (Hanna-Rose and Han, 2002). We obtained *egl-26* deletion allele *tm1244* from the National Bioresource Project at Tokyo Women's Medical University, Japan. *tm1244* deletes 603 base pairs of *egl-26*, including the entire H-box domain. The deletion results in a frame shift that introduces a leucine codon and a stop codon immediately after codon 129. Thus, *tm1244* is predicted to encode a protein truncated after a total of 130 amino acids without any of the domains conserved in the NlpC/P60 family (Fig **2-1**). *egl-26(tm1244)* animals have a qualitatively and quantitatively similar phenotype (66% Egl, n=200; 76% Cog, n=62) to the EMS generated alleles (Hanna-Rose and Han, 2002). To determine if *egl-26(tm1244)* is a complete loss-of-function allele, I scored animals heterozygous for *egl-26(tm1244)* and the deficiency *nDf3*, which includes the *egl-26* locus. *egl-26(tm1244)/ nDf3* animals have an egg-laying defect (66% Egl n=41)

indistinguishable from *egl-26(tm1244)* (above). This suggests that *egl-26(tm1244)* is likely a near-null allele. The incomplete penetrance of this *egl-26* deletion allele suggests that EGL-26 promotes, but is not always required, to establish proper vulval morphology.

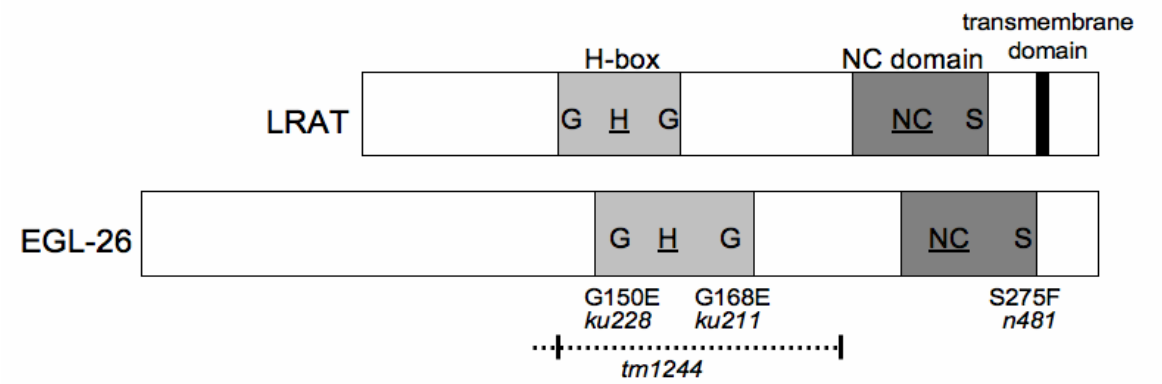

Figure **2-1**: Schematic comparing protein motifs between EGL-26 and LRAT. The positions of the conserved H-box (light grey boxes) and NC domains (dark grey boxes) that characterize eukaryotic NlpC/P60 superfamily proteins are indicated. The defining histidine (H) and asparagine/cysteine (NC) are underlined in the H-box and NC domain, respectively. The thick black line represents the LRAT transmembrane domain. EMS induced alleles of *egl-26* (*ku228*, *ku211* and *n481*) and the substitution mutations that they cause are indicated beneath the conserved amino acids that they alter. The line below the EGL-26 sequence represents the position of the out-of-frame deletion in the *tm1244* allele, which is predicted to encode a truncated protein that includes residues 1 to 129 plus a leucine prior to the stop codon introduced by the frame shift. Sequence identity/similarity between EGL-26 and LRAT is 34/69% in the H-box and is 38/59% in the NC domain.

Phenotypic observation suggested that in *egl-26* mutants vulF resembled the underlying cell called vulE (Hanna-Rose and Han, 2002). Since vulF, and its ventral neighbor vulE, comprise the primary vulval lineage and both are descendents of P6.p, I wanted to test if there were any fate specification defects in these closely related cells.

Previous work using a nuclear localized *egl-26*p::GFP(NLS) transcriptional fusion as a marker revealed no evidence that vulF was adopting a vulE fate in the mutants (Hanna-Rose and Han, 2002). Here I present further evidence that no fate transformation has occurred. Although few genes are expressed differentially in vulE versus vulF (Inoue et al., 2002), *lin-3::*GFP is expressed in vulF but not vulE (Chang et al., 1999) and *zmp-1::*GFP is expressed in vulE but not vulF (Wang and Sternberg, 2000). I found that both markers retain their expected expression pattern in *egl-26* mutants (Fig **2-2**).

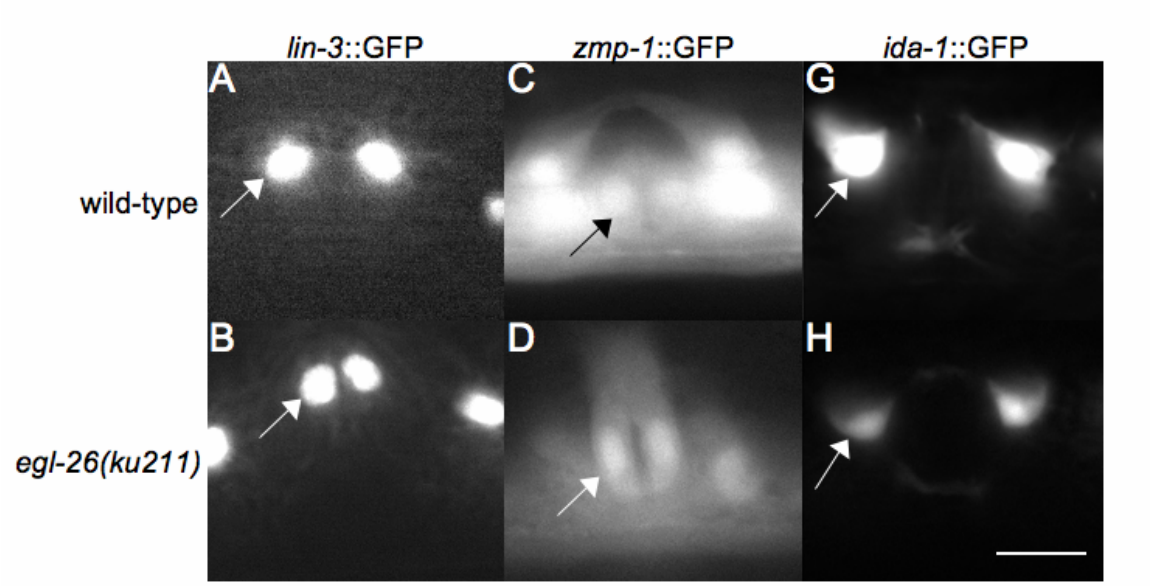

Figure **2-2**: Vulva cells are properly specified and vulF retains some function in *egl-26(ku211)*. Expression of (A,B) *lin-3*::GFP in vulF (arrows), (C,D) *zmp-1*::GFP in vulE (arrows), and (E,F) *ida-1*::GFP in uv1 cells (arrows), which are induced by signaling from vulF, in wild type (top row) and *egl-26(ku211)* mutants (bottom row). Scale bar, 10 μm.

I have also used a functional assay to examine vulF fate. A *lin-3* EGF signal originating from vulF induces four uterine cells to adopt an uv1 cell fate. In the absence of vulF, uv1 fate specification is compromised (Chang et al., 1999). To assay uv1 fate I used *ida-1*::GFP, which within the uterus is brightly expressed only in the specified uv1 cells (Zahn et al., 2001). *ida-1*::GFP is expressed normally in all *egl-26(ku211)* animals (Fig **2-2)**, not only confirming that *lin-3* is likely expressed at the proper time in vulF, but also demonstrating that signaling interactions between the mutant vulF cell and neighboring cells are functional. Thus, in combination with previously reported data, I find no evidence of a fate specification defect in *egl-26* mutants. Instead of affecting vulF fate, mutation of *egl-26* likely has a more specific effect on vulF morphogenesis.

Since EGL-26 is distinctly localized to the apical membrane of vulE in the vulval epithelium (Hanna-Rose and Han, 2002) and mutation of *egl-26* leads to morphological abnormalities in vulF, we also examined vulF cell polarity in the mutants. PAR-6 is an apical surface protein and is important for establishing epithelial cell polarity (Hung and Kemphues, 1999; Hurd and Kemphues, 2003). Rasika Kalamegham used anti-PAR-6 antibody to examine whether the apico-basal polarity of vulF is compromised in *egl-26* mutants. PAR-6 localizes to the apical surface of vulF in mutants, even at late stages of morphogenesis when the morphology defect is obvious (Fig **2-3**). This corroborates the observation that AJM-1 is properly localized to the apical junctions in vulval cells of *egl-26* mutants (Hanna-Rose and Han, 2002). Taken together, we conclude that loss of EGL-26 does not disrupt polarization or cause mis-localization of other apical markers despite the EGL-26 sub-cellular localization at the apical membrane.

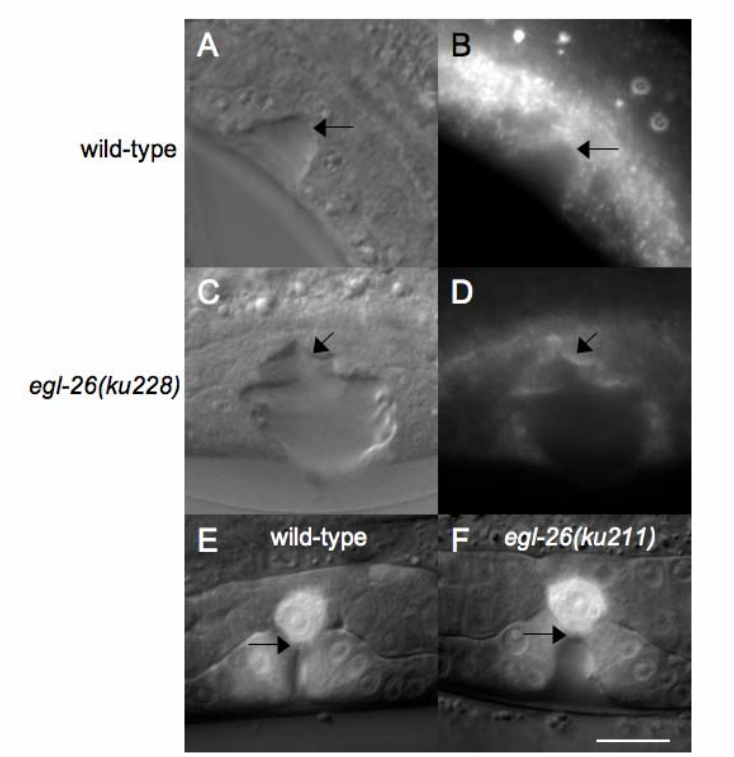

Figure **2-3**: Cell polarity and AC invasion are normal in *egl-26(ku211)*. (A,C) Differential interference contrast (DIC) photomicrographs and (B,D) corresponding fluorescent images of animals stained with anti-PAR-6 antibody, a marker for the apical surface of epithelial cells (Hung and Kemphues, 1999; Hurd and Kemphues, 2003). Apical localization is obvious in (A,B) N2 wild-type animals at the early L4 stage and throughout L4 (not shown, (Hurd and Kemphues, 2003). Similarly, PAR-6 is still apically localized in *egl-26(ku211)* mutants at the late L4 stage (C,D) after the morphology defect is obvious. (E,F) Fluorescent images overlaid onto DIC images of the same animal. The AC comes into close contact with the vulva lumen (arrows) and sits in the middle of vulF precursor cells in both (E) N2 and (F) *egl-26(ku211)* animals. Scale Bar, 10μm.

In wild-type animals, the uterine AC invades the space that will become the lumen in the vulF toroid and subsequently fuses to the uterine seam cell (Newman et al., 1996; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003). To determine if a space within the future vulF cell was initially invaded and occupied by the AC in *egl-26* mutants, I used *cdh-3*::GFP, which allows clear visualization of the boundaries of the AC throughout L3 and into early L4 larval stages when the AC normally fuses to the uterine seam cell (Hanna-Rose and Han, 1999; Sherwood and Sternberg, 2003). AC invasion occurs normally in *egl-26(ku211)*; the AC clearly makes contact with the developing vulval lumen (Fig **2-3**). The AC also fuses with the uterine seam cell (data not shown) as seen in wild-type animals.

No lumen is evident within the vulF toroid in *egl-26* mutants as observed by either DIC microscopy or the apical marker AJM-1::GFP (Hanna-Rose and Han, 2002). However, these methods do not allow a clear view of the membrane structure of vulF. To look more closely at the shape of the vulF cell, I used TAT-2::GFP, which is expressed at the plasma membrane in vulE and vulF (Lyssenko et al., 2008). Reconstructing a 3D image from a Z-series stack of confocal images, I was able to visualize lumen formation in *egl-26* mutants. The initiation of vulval lumen formation appears wild type (Fig **2-4A,B)**. This was not unexpected given the normal morphology observed during early L4 using DIC microscopy. Surprisingly, during mid-L4 when the morphology defect is observed, the vulF toroid in *egl-26* mutants did have a lumen (Fig **2-4D´´**). However, the size and shape of the lumen is variable and it never has the wide-open shape apparent in wild type animals (Fig **2-4C´´**). It appears that the vulF cells collapse after withdrawal of the AC into the uterus. The shape of the vulF cell itself is clearly perturbed, and the

apical surface of the cell appears to collapse into the central lumen, despite normal AC invasion (Fig **2-4D´**). I conclude that *egl-26* acts late in morphogenesis of vulF to maintain the proper cell shape and lumenal structure.

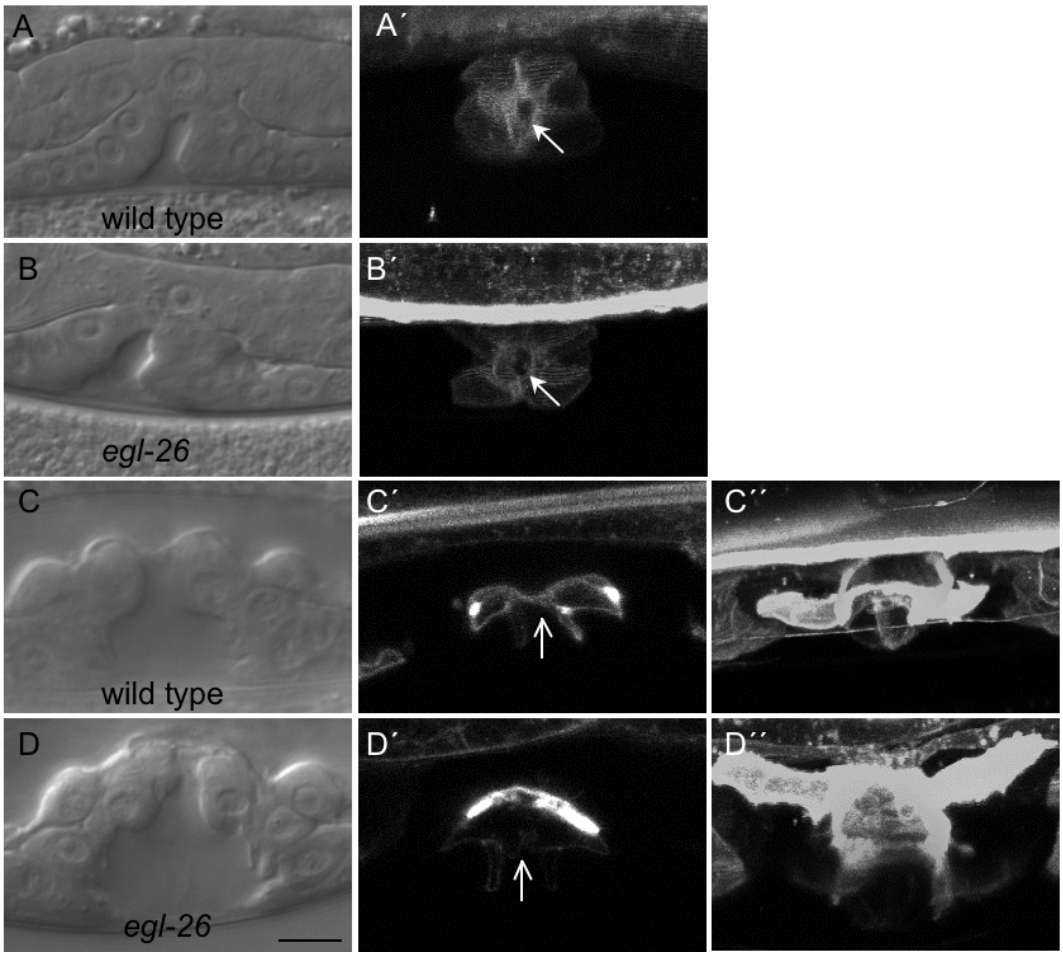

Figure **2-4**: *egl-26* mutants have a partially collapsed lumen in vulF. Left panels are DIC photomicrographs of (A,C) wild type and (B,D) *egl-26(tm1244)*. (A,B) Early L4 animals. (A´,B´) Dorsal view of 3D reconstructions of Z-series confocal images of TAT-2::GFP transgenic animals. Initiation of dorsal lumen formation is the same in wild type (A) and *egl-26* (B). Arrows indicates lumen. (C,D) Mid-L4 stage. (C´,D´) Individual stack from Z-series in midline plane indicating shape of vulF cell. Arrow indicates un-collpased (C´) or collapsed (D´) vulF structure in wild type and *egl-26*, respectively. (C´´,D´´) Dorso-lateral view of 3D reconstructions of Z-series confocal images of TAT-2::GFP transgenic animals. (C´´) Wide open dorsal lumen apparent in wild type. (D´´) Partially collapsed dorsal lumen in *egl-26* mutant. Scale bar: 5μm.

Putative catalytic residues of the H-box and NC domain are essential for EGL-26 function

In order to determine the significance of the sequence homology between EGL-26 and LRAT, I assessed the importance of the conserved residues in the H-box and NC domain to the biological function of EGL-26. Via site-directed mutagenesis, I substituted alanine for histidine 166 and cysteine 261, which are conserved among the family at large (Anantharaman and Aravind, 2003) and are essential for LRAT catalytic activity (Jahng et al., 2003; Mondal et al., 2000; Mondal et al., 2001; Xue and Rando, 2004) (Fig **2-5**). I also mutated His178, which is conserved between LRAT and EGL-26 but is not important for LRAT catalytic activity (Mondal et al., 2001) to demonstrate that any effects are specific to the putative catalytic residues. I performed the mutagenesis in the context of a GFP fusion protein. I assayed function of the transgene via restoration of egg-laying ability in an *egl-26(ku228)* mutant background and simultaneously monitored expression and sub-cellular localization of the fusion protein via visualization of GFP.

EGL-26::GFP robustly rescued the egg-laying defect of *egl-26(ku228)* animals (Fig **2-5**)**.** However, EGL-26(H166A)::GFP and EGL-26(C261A)::GFP did not (Fig **2-5**)**.** In contrast, mutation of the non-catalytic residue H178 had no effect on EGL-26 protein activity; EGL-26(H178A)::GFP rescued the *egl-26(ku228)* Egl phenotype as efficiently as EGL-26::GFP (Fig **2-5**). While I can observe differences in intensity of expression among individuals within a transgenic strain as expected when using extrachromosomal arrays, I see no differences between the strains. After examining more than 75 animals from each strain, I cannot detect any significant differences in expression

among the functional and non-functional transgenes. I conclude that the non-functional GFP fusion proteins are expressed with the same intensity in the vulva and exhibit the same sub-cellular localization patterns as the functional fusions (Fig **2-6**).

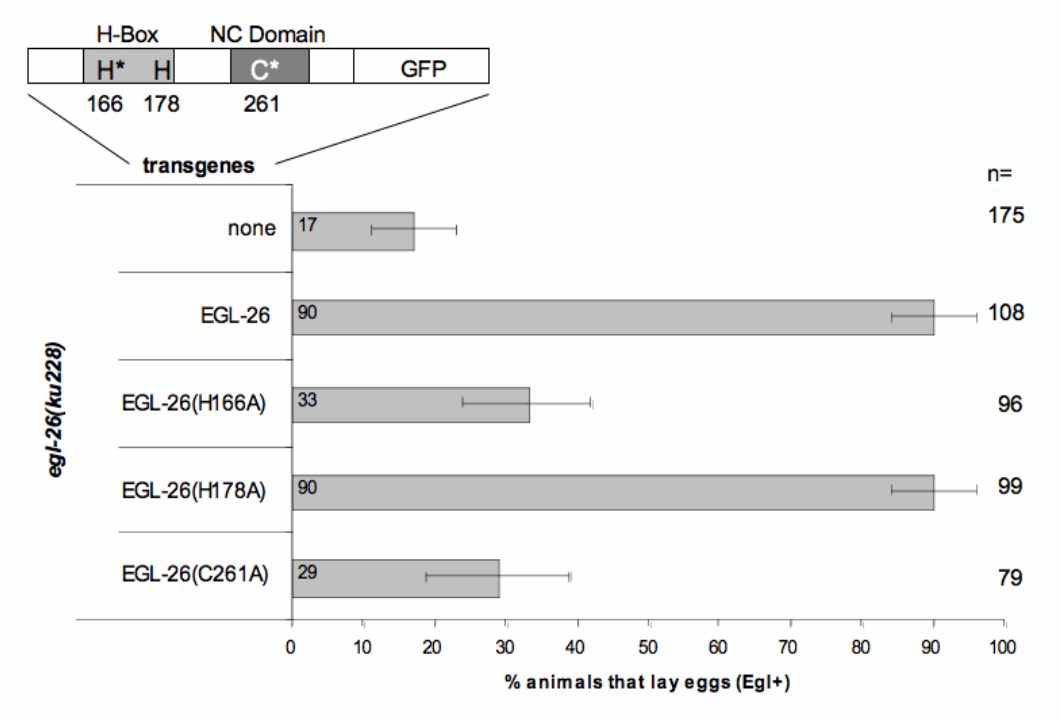

Figure **2-5**: The residues predicted to be important for catalysis based on homology to LRAT are required for EGL-26 function *in vivo*. Schematic showing mutated residues in EGL-26 and site of GFP fusion, asterisks indicate residues conserved in LRAT that are important for catalysis. Histogram showing egg-laying percentage of *egl-26(ku228)* mutant populations carrying transgenes encoding wild-type EGL-26::GFP or EGL-26::GFP protein with the indicated alanine substitution mutation. Actual percentage is cited within each bar. Error bars indicate a 95% confidence interval. Sample sizes (n) are indicated to the right of each bar.

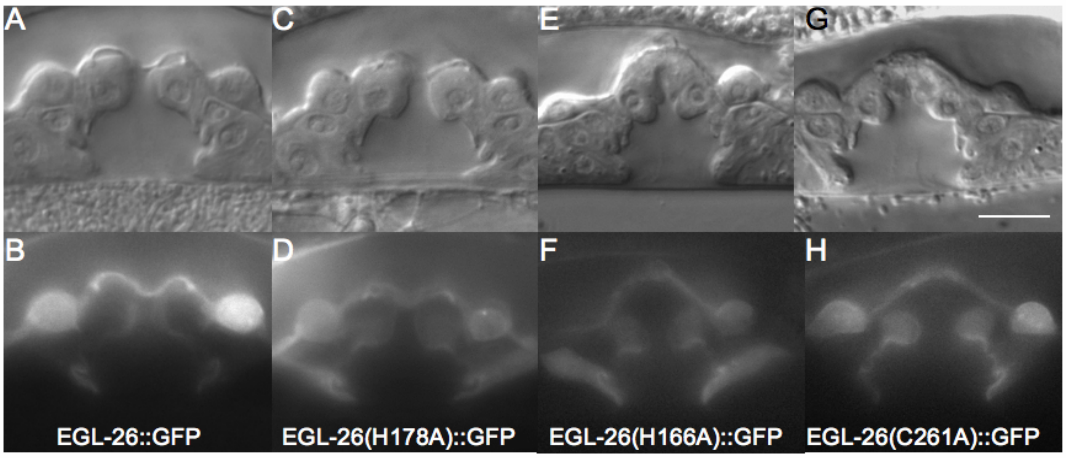

Figure **2-6**: Mutation of putative catalytic residues in EGL-26 does not alter expression. (A,C,E,G) DIC photomicrographs and (B,D,F,H) corresponding fluorescent images of *egl-26(ku228)* mutants expressing the indicated extra-chromosomal arrays at mid-L4-stage. (A,B) *psEx84*[EGL-26::GFP] (C,D) *psEx36* [EGL-26(H178A)::GFP]. (E,F) *psEx37*[EGL-26(H166A)::GFP]. (G,H) *psEx43*[EGL-26(C261A)::GFP]. All GFP fusion proteins are concentrated at the apical membrane. Scale Bar, 10μm.

Serine 275 is important for EGL-26 function in vivo

The EMS-induced *egl-26* allele, *n481*, is a substitution of phenylalanine for conserved serine 275 near the end of the NC domain (Fig **2-7**). EGL-26(S275F)::GFP has no activity as assayed by its ability to rescue the Egl defect of *egl-26(ku211)* animals (Fig **2-7**). Furthermore, EGL-26(S275F)::GFP localizes to the cytoplasm rather than at the apical membrane (Fig **2-**8). I analyzed the effects of additional serine 275 substitution mutations on EGL-26 function and localization. I created the phosphomimetic mutation

S275E because predictions using NetPhos 2.0 (Blom et al., 1999) suggest that serine 275 is highly likely to be phosphorylated. In addition, I substituted alanine and threonine residues instead of the bulky hydrophobic phenylalanine found in the mutant.

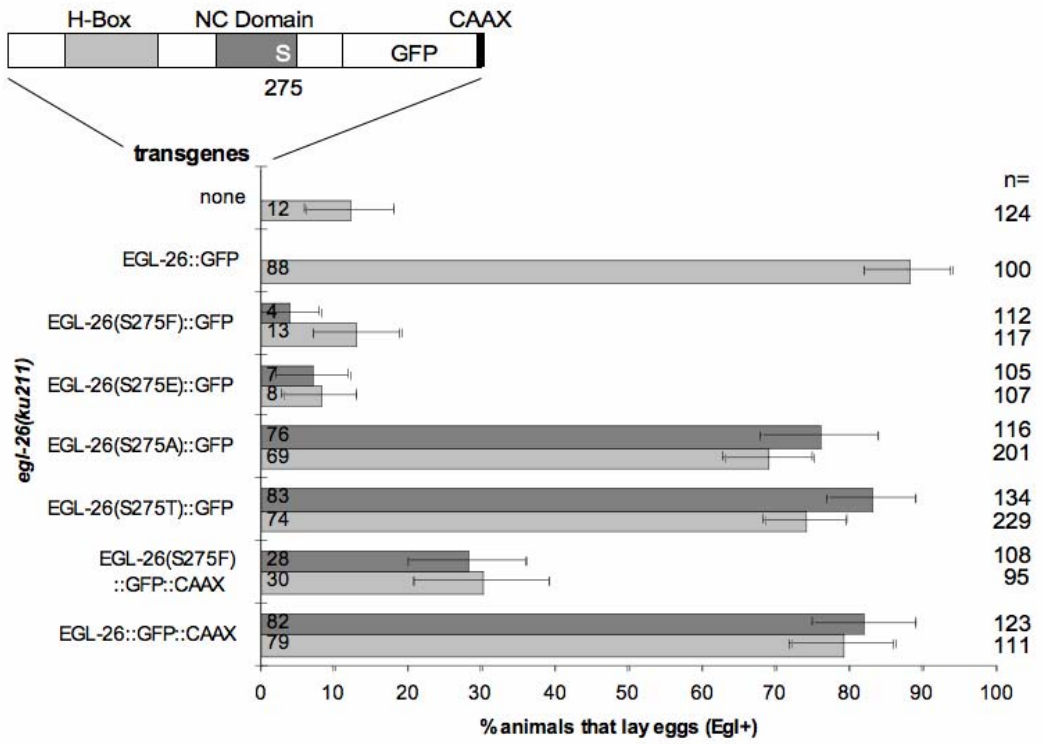

Figure **2-7**: Serine 275 is important for EGL-26 function and membrane localization correlates with function. Schematic showing mutated Ser275 in EGL-26 and the site of the GFP and CAAX fusions. Histogram showing egg-laying percentage of *egl-26*(*ku211*) mutant populations carrying transgenes encoding wild-type EGL-26::GFP or the indicated EGL-26::GFP variant. The two bars represent separate transgenic lines generated for each construct. EGL-26(S275F) is mis-localized (Fig **2-8**C,D) and does not rescue the egg-laying defect of *egl-26(ku211)*. Restoring membrane localization to EGL-26(S275F)::GFP with a CAAX prenylation signal (Fig **2-8**E,F) increases its egg-laying ability to at least twice that of EGL-26(S275F) alone, resulting in a partial rescue of the Egl phenotype. Similar to EGL-26(S275F), EGL-26(S275E) also does not rescue the mutant. EGL-26::GFP::CAAX retains the egg-laying ability of EGL-26::GFP. EGL-26(S275A) and one of the two lines of EGL-26(S275T) partially rescue the mutant, while the other line of EGL-26(S275T) rescues egg-laying ability as efficiently as EGL-26::GFP. Error bars indicate a 95% confidence interval. Sample sizes (n) are indicated to the right of each bar.

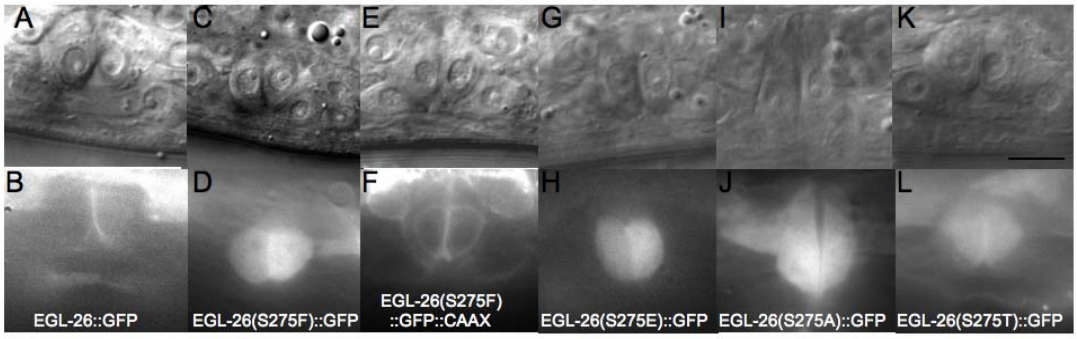

Figure **2-8**: Mutation of serine 275 can alter the sub-cellular localization of EGL-26. (A,C,E,G,I,K) DIC photomicrographs and (B,D,F,H,J,L) corresponding fluorescent images of two lateral vulE cells at the mid-L4-stage. (A,B) *kuEx90*[EGL-26::GFP] is concentrated at the apical membrane of vulE. (C,D) *psEx112*[EGL-26(S275F)::GFP] has a diffuse, generally cytoplasmic localization. (E,F) *psEx25*[EGL-26(S275F)::GFP::CAAX] is membrane localized, but not apically concentrated. (G,H) *psEx77*[EGL-26(S275E)::GFP] has a diffuse, generally cytoplasmic localization. (I,J) *psEx85*[EGL-26(S275A)::GFP] is partially concentrated at the apical membrane but is also present in cytoplasm. (K,L) *psEx86*[EGL-26(S275T)::GFP] is partially concentrated at the apical membrane but is also present in cytoplasm. The localization shown here was consistent for all transgenic lines tested. Scale Bar, 10 μm.

Insertion of a glutamate did not restore function or sub-cellular localization to EGL-26. EGL-26(S275E)::GFP cannot provide egg-laying activity in the rescue assay (Fig **2-7**), and this mutant was not localized to the membrane (Fig **2-8H**). However, both the threonine and alanine substitution mutations were permissive for function. Transgenes encoding these proteins provided between 78% and 94% of the function provided by a wild-type protein (Fig **2-7**). These proteins were also slightly enriched at the apical membrane relative to EGL-26(S275F) (Fig **2-8J,L**), which was never

concentrated at the apical membrane but was easily visible in the cytoplasm (Fig **2-8D**). Although EGL-26(S275A)::GFP and EGL-26(S275T)::GFP regain a slight enrichment at the apical membrane, each is still overabundant in the cytoplasm relative to EGL-26::GFP (Fig **2-8B**). Phosphorylation of Ser 275 does not play a role in EGL-26::GFP membrane localization or function based on the above data. I conclude that serine 275 plays a role in membrane localization and mutation of this residue likely affects protein function more generally as well.

Membrane localization plays a role in EGL-26 function

Intriguingly, when the H-box and NC domains were first recognized, they were noted to occur in combination with predicted transmembrane domains (Hughes and Stanway, 2000). LRAT has a C-terminal transmembrane domain that results in a topology with the catalytic domain in the cytoplasm (Moise et al., 2007; Ruiz et al., 1999). Hrasls3 has a predicted transmembrane domain, and at least a subset of the cellular protein is associated with membranes (Hajnal et al., 1994; Sers et al., 1997). Unlike the other Hbox/ NC domain-containing eukaryotic proteins, EGL-26 has no strongly predicted alpha-helical, membrane-spanning region (Claros and Heijne, 1994; Krogh et al., 2001; von Heijne, 1992). However, EGL-26 is specifically localized at or near the apical membrane in cells where it is expressed (Hanna-Rose and Han, 2002).

These observations, coupled with the data indicating that serine 275 plays a role in localization, led us to hypothesize that membrane localization may be required for

function and that perhaps EGL-26(S275F) did not function because it was available at the membrane only a fraction of the time, if at all. Based on this hypothesis, we predicted that if we could increase the fraction of EGL-26(S275F) at the membrane, we could increase its activity. To test this prediction, Rasika Kalamegham incorporated a CAAX motif (CNIM) at the C-terminus of EGL-26(S275F)::GFP (Fig **2-7**). The C-terminal CAAX motif is a prenylation signal and results in lipid modification and subsequent membrane localization of the covalently linked protein (Maurer-Stroh et al., 2003). I created the transgenic lines and found that addition of the CAAX motif to EGL-26(S275F)::GFP induced localization to the membrane but not specifically to the apical surface, as expected (Fig **2-8F**). Interestingly, the plasma membrane bound EGL-26(S275F)::GFP::CAAX protein had increased activity and an egg-laying percentage at least twice that of EGL-26(S275F)::GFP (Fig **2-7**). As a control, I also confirmed that tethering the GFP to the membrane via its C-terminus had no effect on EGL-26::GFP activity (EGL-26::GFP::CAAX, (Fig **2-7**). I conclude that membrane localization contributes to EGL-26 function and serine 275 plays a role in localization.

DISCUSSION

Primary vulval fates are normally specified in *egl-26* mutants but late aspects of morphogenesis are abnormal

In an *egl-26* mutant, the most obvious defect is the distorted shape of the polarized epithelial vulval cell called vulF. Our analysis of the apical markers PAR-6 (Fig **2-3**) and AJM-1 (Hanna-Rose and Han, 2002) demonstrate that vulF is properly polarized in *egl-26* mutants. Despite the observation that vulF somewhat resembles vulE in *egl-26* mutants (Hanna-Rose and Han, 2002), expression of vulE-specific and vulF-specific markers are restricted to the expected cell types, and uv1 fate is induced (Fig **2-2**). Early morphogenesis of vulF during AC invasion is normal in the mutants (Fig **2-3**), but analysis of later cell shape using TAT-2::GFP demonstrates that vulF does have a central lumen, although it's often much smaller than that observed in wild type (Fig **2-4)**. It appears that perhaps the apical membrane of vulF collapses after the AC withdraws into the uterus. Taken together, my results demonstrate that 1) vulF fate specification, 2) early morphogenesis of the vulF cell, including establishment and maintenance of cell polarity, 3) vulF-uterine cell-cell signaling capabilities are all maintained in the *egl-26* mutant, and 4) *egl-26* mutants have a dorsal vulval lumen but vulF at least partially collapses inward, and therefore EGL-26 likely plays a role in maintaining the dorsal lumenal structure. I conclude that *egl-26* acts quite specifically in a late stage of vulF cell morphogenesis. My conclusion is consistent with the observation that EGL-26::GFP

expression is first observed in vulE relatively late in morphogenesis, during the mid-L4 stage (Hanna-Rose and Han, 2002).

EGL-26 acts to ensure the fidelity of vulF morphogenesis

All EMS-induced alleles of *egl-26* cause missense mutations that result in an incompletely penetrant vulF cell morphology defect. After analyses of the EMS-induced alleles, it was unclear if the incomplete penetrance was due to weak loss-of-function effects or due to a function for EGL-26 in ensuring consistent results during morphogenesis, as opposed to an absolute requirement for the protein. Attempts to address this question using RNAi to phenocopy a null or loss-of-function phenotype have failed. We observe only a 4% Egl phenotype upon RNAi of *egl-26*, and genome-wide screens have similarly failed to reveal any *egl-26(RNAi)* effect (Kamath et al., 2003; Rual et al., 2004; Sonnichsen et al., 2005). I have now addressed the question of gene function by examining a deletion allele of *egl-26*. *egl-26(tm1244)* behaves genetically like a null allele and completely lacks the sequences that define the NlpC/P60 family, specifically residues H166 and C261, which are critical for function as demonstrated by my mutagenesis experiments. Thus, any protein encoded by *egl-26(tm1244)* is unlikely to retain function. Yet, *egl-26(tm1244)* is also incompletely penetrant. I suggest that although EGL-26 promotes appropriate morphogenesis of vulF, it is unlikely to be absolutely required for normal vulF morphogenesis. Interestingly, we have observed that

two of the EMS-derived mutations (*ku228* and *ku211*) have a more penetrant Egl phenotype than *egl-26(tm1244)*. No *egl-26* alleles have any dominant properties and all are rescued by expression of the wild-type protein. Nonetheless, the unusual higher penetrance of *ku228* and *ku211* relative to the putative molecular null *tm1244* hint at the possibility of a more complicated genetic function for these alleles in vulval morphogenesis.

EGL-26 is an NlpC/P60 protein and is likely to have a biochemical function similar to LRAT

egl-26(ku228), the most penetrant of the EMS-induced alleles, affects glycine residue 150 (Fig **2-1**), which is conserved in 95% of proteins in the LRAT subfamily (Anantharaman and Aravind, 2003). The EMS-induced *egl-26* alleles *ku211* and *n481* encode proteins with mutations at G168 and S275, which are both perfectly conserved in the LRAT subfamily but not in the wider superfamily. These observations suggest that G150, G168 and S275 may be important for function in the LRAT subfamily of eukaryotic proteins. The positions of these mutations highlight the importance of the likely LRAT-like biochemical activity to the developmental function of EGL-26 and suggest that all *egl-26* alleles result in a significant reduction-of-function.

NlpC/P60 superfamily members perform an array of functions in prokaryotes but aren't as well studied in higher eukaryotes (Anantharaman and Aravind, 2003). I have shown that the residues His166 and Cys261, which are conserved throughout the larger

superfamily and are required for catalysis in LRAT (Anantharaman and Aravind, 2003; Xue et al., 2004), are essential for EGL-26 function *in vivo*. My results specifically illustrate the importance of the catalytic residues and an LRAT-like catalytic activity in an animal developmental morphogenesis context.

EGL-26 membrane localization is correlated with function

EGL-26(S275F)::GFP is mis-localized to the cytoplasm and does not function in the Egl rescue assay. Interestingly, a mutation of the corresponding serine residue in LRAT to arginine (S175R) has been identified in humans with early-onset retinal dystrophy (Thompson et al., 2001). Furthermore, LRAT(S175R) retained no enzymatic activity, indicating that Serine 175 is essential for LRAT activity (Thompson et al., 2001). Localization of LRAT(S175R) was not reported. However, this protein retains its transmembrane domain and so would presumably be localized to the membrane if expressed.

Substitution of the hydrophobic phenylalanine or phosphomimetic charged glutamic acid for serine 275 cannot support protein function, but localization of EGL-26(S275F)::GFP to the membrane using a CAAX motif increases its function. This suggests that membrane localization is important for EGL-26 function and that EGL-26(S275F)::GFP is at least partially functional if available at the membrane. Interestingly, substitution of the smaller residues alanine and threonine at position 275 can support protein function. I reason that the likely effect of S275F is to alter protein

structure, thereby perturbing localization as well as activity as opposed to specifically affecting membrane affinity. This indicates that the mechanism of protein localization for EGL-26 is likely to be via a protein-protein interaction as opposed to a cryptic membrane-spanning region or lipid modification of a specific linear amino acid motif. This conclusion is supported by our unpublished observation that the N-terminal third, the middle third and the C-terminal third of EGL-26 are each insufficient to direct membrane localization of EGL-26. Instead, I suggest that EGL-26 is unique in the LRAT sub-family of NlpC/P60 proteins in its mechanism for attaining membrane localization. I also conclude that membrane localization correlates with function, as only mutants that exhibit membrane localization function in the Egl rescue assay and restoration of membrane localization to a cytosolically localized mutant increases protein function.

Materials and Methods

Maintenance and culture of C. elegans strains

Unless otherwise stated, strains were grown under standard conditions at 20° (Brenner, 1974). We used the following strains, alleles and transgenes: N2 wild type; PS4308 *syIs107*[*unc-119*(+) + *lin-3*(*delta-pes-10*)::GFP]; MH1371 *kuIs38*[*dpy-20*(+) + *cdh-3*::GFP]; LG II: *egl-26(ku211, ku22, n481) and egl-26(tm1244)* (obtained from the National Bioresource Project, Tokyo Women's Medical University, Japan), MT681

nDf3/lin-31(n301) bli-2(e768) (Greenwald and Horvitz, 1980), BL5715 *inIs179*[*ida-1*::GFP] (Zahn et al., 2001); CB5584 *mIs12*[*myo-2*::GFP, *pes-10*::GFP, *F22B7.9*::GFP]; LG III: *unc-119(ed3)*; LG IV: PS3239 *dpy-20(e1282) syIs49*[dpy-20(+) + *zmp-1*::GFP)] (Inoue et al., 2002); LG V: *him-5(e1490).* Additional genetic information is available at http://www.wormbase.org.

Cloning and Genetics

To create vectors encoding EGL-26 mutant proteins, we performed site-directed mutagenesis of pWH15[EGL-26::GFP] (Hanna-Rose and Han, 2002) by recombinant PCR. We used primers at the site of mutagenesis (Table **2-1**), and the following outside primers

Table **2-1**: Mutagenesis primers

Construct	Mutagenesis primers
pH166A [EGL-26(H166A)::GFP]	GGTGTAAAATTCTATGCCAGTGGAATTTATGC GCATAAATTCCACTGGCATAGAATTTTACACC
pH178A [EGL-26(H178A)::GFP]	GGAATGTGCTACGCTTTTGTCTGC GGAATGTGCTACGCTTTTGTCTGC
pC261A [EGL-26(C261A)::GFP]	CGTTGCAACGCCCAACATTTCTCATCGG CCGATGAGAAATGTTGGGCGTTGCAACG
pS275F [EGL-26(S275F)::GFP]	CCCATTCTTTTATGACATGAC GTCATGTCATAAAAGAATGGG
pS275A [EGL-26(S275A)::GFP]	GGCGTCCCATTCGCTTATGACATGAC GAAGTCATGTCATAAGCGAATGGGAC
pS275E [EGL-26(S275E)::GFP]	GGCGTCCCATTCGAATATGACATGACTTC GAAGTCATGTCATATTCGAATGGGACGCC
pS275T [Egl-26(S275T)::GFP]	GGCGTCCCATTCACTTATGACATGAC GAAGTCATGTCATAAGTGAATGGGAC

for H166 and H178 recombinant PCR products:

PflM I F TTCGATGATCCACCAATTGG

Sac I R CAAAATTTGCCGAGCTCGGC

for C261 and S275F recombinant PCR products:

Sac I F GTCGTCGACGAGCTCGGCAAATTTTGAGATTTACC

BamH I R CGGGATCCCGAAGAAGTACTGCTGCTCGC

for S275E, S275A and S275T recombinant PCR products:

COG-4 5' SEQ GTCTCGTGACCTCATCAGCC

GFP 3' past Nco1 GTAGTGACAAGTGTTGGC

The resulting PCR products were digested with PflM I and Sac I, Sac I and BamH I or Sac I and Age I and ligated into the vector pWH15, from which the wild-type fragment had been removed.

To create a plasmid encoding EGL-26(S275F)::GFP::CAAX, we digested pWH15(S275F) with Xho I and Apa I and isolated two fragments (a 5 kilobase pair Xho I fragment and a 4 kilobase pair Apa I-Xho I). We also digested vector GFP-PM (Portereiko and Mango, 2001) with Xho I and Apa I to isolate a fragment encoding the C-terminus of GFP with a CAAX motif. We ligated this fragment to the 4 kilobase pair fragment from pWH15, digested the product with Xho I and inserted the 5 kilobase pair Xho I fragment from pWH15(S275F) to create pRK3[EGL-26(S275F)::GFP::CAAX].

To create a construct encoding EGL-26::GFP::CAAX, we digested pRK3 and pWH15 with Apa I and BamH I. We isolated a 1.8 kilobase pair fragment from pRK3 containing the GFP::CAAX sequence, but without the S275F mutation. We then

ligated this fragment to an 8.9 kilobase pair fragment isolated from pWH15 after digestion to create pKE4[EGL-26::GFP::CAAX].

We sequenced each construct and created transgenic strains using germline transformation (Mello et al., 1991) (Table **2-2**). All constructs were co-injected with unc-119+ at 60ng/µl (Maduro and Pilgrim, 1995).

Table **2-2**: Transgenic Lines

Construct	Genotype injected	Concentration (ng/µl)	Transgene
pH166A[EGL26(H166A)::GFP]	*unc-119(ed3)*	20	psEx37
pH178A[EGL26(H178A)::GFP]	*unc-119(ed3)*	20	psEx36
pC261A[EGL26(C261A)::GFP]	*unc-119(ed3)*	20	psEx43
pWH15 [EGL-26::GFP]	*egl-26(ku228)*	15	psEx84
pS275F[EGL-26(S275F)::GFP] Line 2	*egl-26(ku211)*	10	psEx112
pS275F[EGL-26(S275F)::GFP] Line 4	*egl-26(ku211)*	10	psEx113
pS275E[EGL-26(S275E)::GFP] Line 1	*egl-26(ku211)*	10	psEx77
pS275E[EGL-26(S275E)::GFP] Line 2	*egl-26(ku211)*	10	*psEx123*
pS275A[EGL-26(S275A)::GFP] Line 1	*egl-26(ku211)*	10	psEx85
pS275A[EGL-26(S275A)::GFP] Line 2	*egl-26(ku211)*	10	psEx111
pS275T[EGL-26(S275T)::GFP] Line 1	*egl-26(ku211)*	10	psEx86
pS275T[EGL-26(S275T)::GFP] Line 2	*egl-26(ku211)*	10	psEx106
pWH15 [EGL-26::GFP]	*egl-26(ku211)*	10	psEx110
pRK3[EGL26(S275F)::GFP::CAAX] Line 1	*unc-119(ed3)*	10	psEx25
pRK3[EGL26(S275F)::GFP::CAAX] Line 2	*egl-26(ku211)*	10	psEx107
pKE4[EGL-26::GFP::CAAX] Line 1	*egl-26(ku211)*	10	psEx64
pKE4[EGL-26::GFP::CAAX] Line2	*egl-26(ku211)*	10	psEx109

Scoring of egg-laying and cell fate phenotypes

To determine if *in vitro* mutagenized EGL-26::GFP fusion proteins could rescue *egl-26* mutants, we placed transgenes (see Table 2) in *egl-26(ku228)* or *egl-26(ku211)* backgrounds by direct injection or by mating and scored for egg-laying ability. We scored animals as egg-laying defective (Egl) if they laid no eggs or less than 20 eggs prior to forming the "bag of worms" phenotype caused by unlaid eggs hatching inside the mother.

To test if *egl-26(tm1244)* behaved as a null, we mated *egl-26(tm1244) mIs12; him-5(e1490)* males to *nDf3/lin-31 bli-2* hermaphrodites. We picked GFP-positive cross progeny and scored for egg-laying ability as previously described. We then discarded data from any animal with Bli progeny, thus including only *egl-26(tm1244)/nDf3* animals but not *egl-26(tm1244)/lin-31 bli-2* animals.

To assay cell fate specification and AC invasion, we mated *egl-26(ku211)*, *him-5(e1490)* males into PS4308 *syIs107*[lin-3::GFP], PS3239 *dpy-20(e1282) syIs49*[*zmp-1*::GFP], BL5715 *inIs179*[*ida-1*::GFP], and MH1371 *kuIs38*[*cdh-3*::GFP)] and examined GFP expression as compared to control strains.

Statistical Analysis

To determine if the percentage of animals that lay eggs is statistically significant between different populations, we used the following equations (Milton, 1992):

$L1 = p - z[p(1-p)/n]^{(1/2)}$ and $L2 = p + z[p(1-p)/n]^{(1/2)}$, where $z = 1.96$ for a 95% confidence interval, *L1* is the lower bound and *L2* is the upper bound, *p* is the egg-laying proportion, and *n* is the sample size. Graphs have error bars representing 95% confidence intervals.

Immunofluorescence

Fixed and permeabilized animals (Bettinger et al., 1996) were incubated in rabbit anti-PAR-6 antibody at a dilution of 1:10 in 2% NGS in 1x Buffer A at 4° for 24 hours. We then washed the animals four times for ten minutes in 1x Buffer A and incubated them in Cy-3 conjugated goat anti-rabbit at a 1:100 dilution for three hours at room temperature. Finally we washed samples for ten minutes four times in 1x Buffer A, twice for one hour and then overnight. We mounted stained animals in 80% glycerol with anti-bleaching agents and visualized by epifluorescence on a Zeiss Axioplan 2.

Microscopy

Images were acquired using a Zeiss LSM 510 confocal microscope or a Zeiss Axioplan 2. Z-series images were acquired every 0.5 μm and were re-constructed into 3D images using ImageJ (Abramoff, 2004). Some images were processed using

Photoshop.

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Chapter 3

A new role for the anchor cell in *C. elegans* vulval tubulogenesis

Introduction

Tubulogenesis and lumen formation are critical to the development of most organs. As detailed in the introduction, development of the vulva and the ventral uterus is coordinated by the inductive cell-signaling activity of a gonadal cell called the anchor cell (AC). Two types of mutants with defective anchor cell behavior, the *unc-6* gene that guides AC migration and the *fos-1* transcription factor, reveal that AC invasion of the vulva is important to form the toroidal shape of the dorsal vulva cell, vulF. Mutations in *unc-6* (Netrin) and its receptor *unc-40* (DCC/Neogenin) cause defects in invasion of the vulva by the AC because the AC is not properly guided to the vulva. In *fos-1* mutants, where the AC cannot breakdown the basement membranes between the gonad and the vulva, the AC fails to invade the vulva. Failure of AC invasion and penetration of vulF in *unc-6*, *unc-40* and *fos-1* mutants results in the absence of the dorsal vulval lumen. By examining GFP markers of dorsal vulval cell fate, I demonstrate that fate specification defects do not account for the aberrant vulF shape. I conclude that the presence of the AC in the center of the developing vulF toroid is required for dorsal vulval lumen formation to complete vulval tubulogenesis.

Results

unc-6 has a specific dorsal vulval morphology defect

The mutant *ku243* was isolated in a screen for egg-laying defective animals associated with vulval morphology defects (Hanna-Rose and Han, 1999; Hanna-Rose and Han, 2002). *ku243* has three distinct and penetrant phenotypes: uncoordinated (Unc), egg-laying defective (Egl), and connection of gonad (Cog) defective (Fig **3-1**). The ventral vulva appears to be morphologically normal, but the dorsal vulva is occluded. vulF is misshapen and exhibits a similar morphology defect to *egl-26*. No connection between the vulval and uterine lumens is apparent. Three-point mapping revealed that *ku243* was near the *mup-2* locus on the X chromosome, 0.9 map units from *unc-6*. Because of the observed Unc phenotype, I tested if *ku243* was allelic to *unc-6* by attempting to rescue the *ku243* mutant phenotypes using an UNC-6::HA rescuing transgene (Wadsworth et al., 1996). Indeed, the Unc, Egl, and Cog phenotypes of *ku243* were rescued by an UNC-6::HA transgene, indicating that *ku243* is an allele of *unc-6* (data not shown).

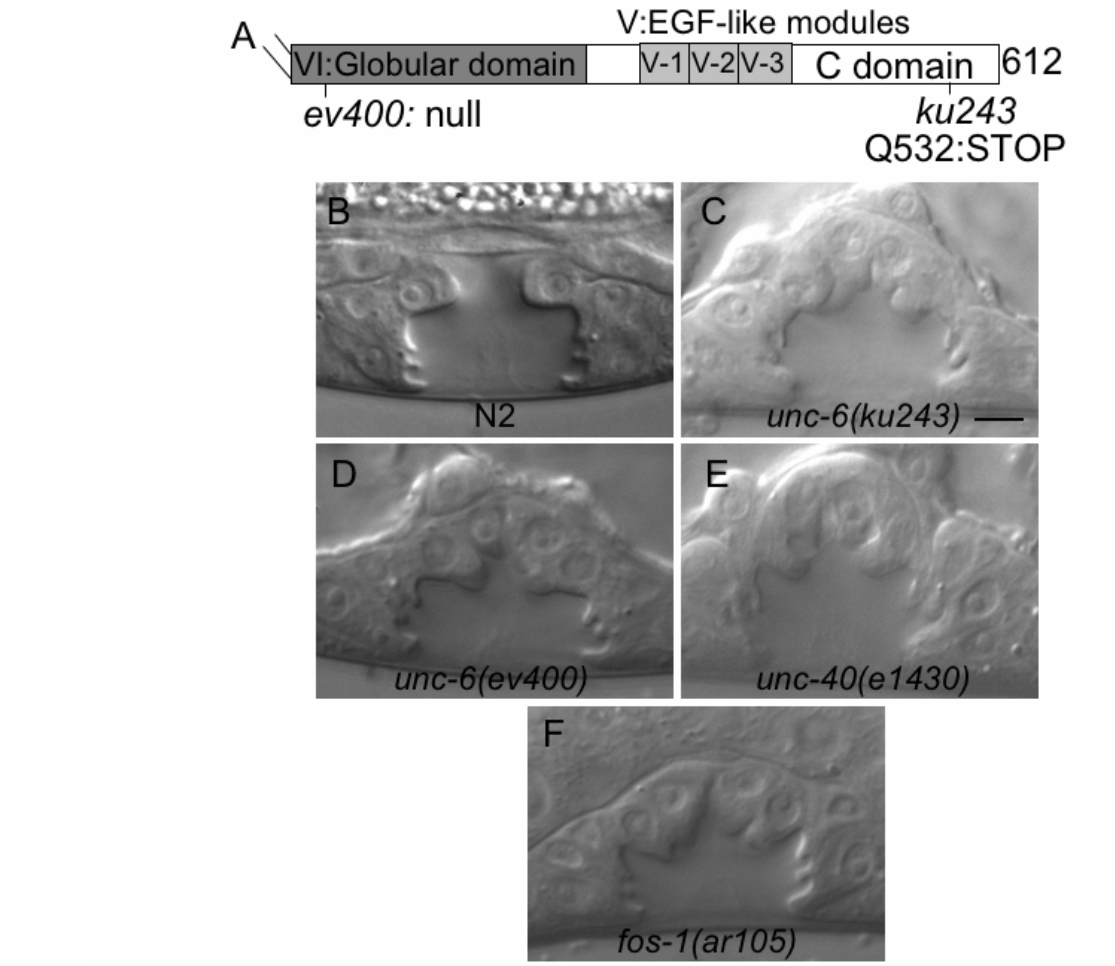

Figure **3-1**: AC invasion defective mutants have vulval morphology defects. (A) Schematic of *unc-6* showing the location of the nonsense mutations in the *ku243* allele and in the *ev400* null allele. Parallel lines at the N-terminus indicate the signal peptide. The darkly shaded box is the globular domain called VI, the lightly shaded boxes indicate the 3 EGF-like modules, and the white box at the end indicates the C domain. (B-F) DIC photomicrographs of mid-L4 vulvae showing (B) normal vulva morphology of wild type and similar morphology defects of (C) *unc-6(ku243),* (D) *unc-6(ev400),* (E) *unc-40(e1430)* and (F) *fos-1(ar105).* The dorsal vulva is misshapen while the ventral vulva appears normal in all mutants. Scale bar: 5 μm.

We sequenced the *unc-6* gene in *ku243* mutants and found a nonsense mutation at codon 532 (Q532X) (Fig **3-1**), which would truncate the protein 80 amino acids prior to the C-terminus. I compared the relative penetrance of the Unc and Cog defects of *unc-6(ku243)* with the null allele *unc-6(ev400)* (Hedgecock et al., 1990). Both the *ku243* and *ev400* alleles have a highly penetrant vulval morphology defect (Table **3-1**). However, *ku243* mutants appear qualitatively less uncoordinated when compared with *ev400* mutants. To quantitate this difference, I tested the ability of the animals to initiate backward movement when lightly stroked on the head with an eyelash. *ku243* animals back up in response to stimulus more readily than the null animals (Table **3-1**). Thus, while *ku243* mutants have a distinctly less Unc phenotype than the null mutants, they have a vulval morphology defect as severe as the null mutants. I conclude that *ku243* is an allele of *unc-6* and that mutation of *unc-6* causes a Cog defect.

Table **3-1**: *ku243* preferentially affects vulva morphology

strains	**% wild-type vulva morphology**[1]	**n**	**% backing**[2]	**n**
N2	100	many	100	25
unc-6(ev400)	16	81	56	25
unc-6(ku243)	20	101	76	25
unc-40(e1430)	43	88	not done	

[1] See Fig **3-1;** [2] Animals that move backward

To determine if *unc-6* is likely affecting vulval morphology via its well characterized function as a cell and axon guidance molecule, I examined the vulval morphology of the *unc-6* receptor mutant *unc-40*. *unc-40* mutants exhibit the vulval

morphology phenotype, although with a lower penetrance than *unc-6* mutants (Fig **3-1**, Table **3-1**).

The *unc-6* / *unc-40* vulval morphology defect is not due to fate mis-specification

Many documented vulval morphology defects arise from aberrant vulva fate specification (reviewed in (Sternberg, 2005). Indeed, there is evidence that mutation in *unc-6* can cause vulval cells to be improperly specified (Wang and Sternberg, 2000). To determine how *unc-6* and *unc-40* affect vulval morphogenesis, I first examined whether vulval cell fate specification is perturbed in the mutants using the null alleles *unc-6*(*ev400)* and *unc-40*(*e1430)* (Chan et al., 1996; Hedgecock et al., 1990). Since the ventral vulva is apparently normal in both *unc-6* and *unc-40*, I concentrated on the dorsal vulval cells, specifically vulE and vulF. I used GFP reporters as markers of vulE and vulF cell fate specification. ZMP-1::GFP is expressed in vulE beginning late in the fourth larval stage (L4) and *lin-3*::GFP is expressed in vulF during mid L4 (Chang et al., 1999; Wang and Sternberg, 2000). These markers are expressed in the correct cell at the expected time in over 90% of the *unc-6* and *unc-40* animals analyzed (Fig **3-2,** Table **3-2**), while wild-type vulval morphology is observed in less than 25% of all *unc-6* mutants and in less than 45% of all *unc-40* mutants. These data support previous reports of some incidence of fate specification defects in these mutants (Wang and Sternberg, 2000).

However, my data clearly indicate that aberrant fate specification is not the underlying cause of the penetrant vulval morphology defect in the majority of animals.

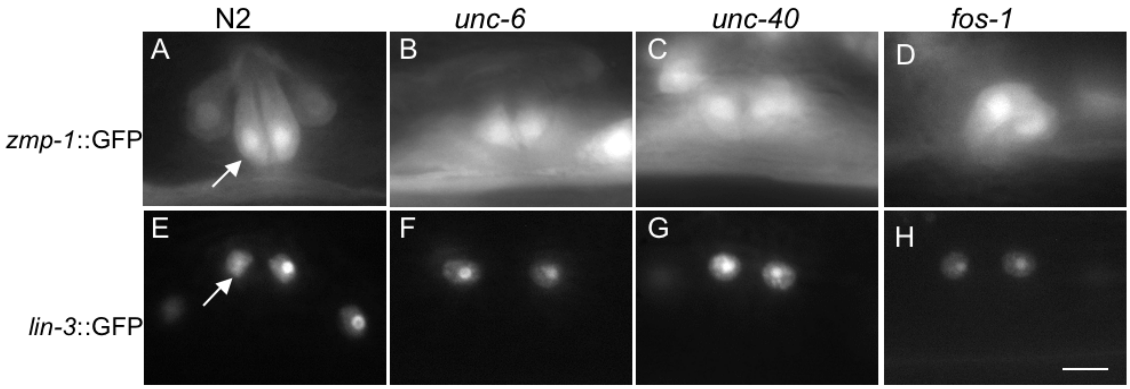

Figure **3-2**: Dorsal vulval fate specification is largely normal in AC invasion defective mutants. (A-D) Expression of *zmp-1*::GFP in vulE (arrow indicates one vulE cell). (E-H) Expression of *lin-3*::GFP in vulF (arrow indicates one vulF cell). Quantification of normal versus abnormal marker expression in Table 2. Scale bar: 5 μm.

Table **3-2**: AC invasion mutants have no fate specification defects

gene	marker	% animals properly specified[1]	n
unc-6		93	97
unc-40	*lin-3*::GFP	94	88
fos-1		100	20
unc-6		97	64
unc-40	*zmp-1*::GFP	92	51
fos-1		100	20

[1] See images, Fig **3-2**

Anchor cell invasion defects cause the *unc-6* vulval morphology defect

UNC-6 guides not only axons, but also various cell migrations (Hedgecock et al., 1987), and Hedgecock *et al.* have reported that the egg-laying defect of *unc-6* mutants is caused by a defect in AC migration. To investigate the possibility that a cell migration defect underlies the vulval morphology defect, I observed both AC invasion at late L3/ early L4 and subsequent morphology during mid L4 in the same animal. In wild-type animals, the AC degrades the basement membranes between the vulva and uterus during L3 and invades the vulva, eventually residing between the presumptive vulF nuclei in late L3/early L4 (Newman et al., 1996; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003). In *unc-6(ev400),* I observed variable levels of AC invasion and have grouped the mutants into 3 phenotypic classes (Fig **3-3**) : 1) no AC breakdown of the

basement membrane or invasion of the vulva because the AC is severely mis-localized in the uterus; 2) the AC degrades the basement membrane and sends processes toward the developing vulval lumen but does not penetrate between the vulF cells to directly contact the vulval lumen; 3) wild-type-like AC invasion. 38% of animals were class 1 showing no AC invasion, 44% of animals were class 2 showing basement membrane breakdown but no significant vulF penetration, and the remaining 18% appeared normal (n=16) (Fig **3-3**). The animals that had normal AC invasion always had a normal vulva at the mid L4 stage. The 82% of animals that had some level of an AC penetration defect at an early stage correlate well with the 84% (Table **3-1**) that have a Cog phenotype at later stages, suggesting that the vulval morphology defect may result from the AC migration defect. Wang, *et. al.* showed that the AC is required at a distance of less than 30 μm from the dorsal vulva during late L3 to function in vulE and vulF fate specification. However, in *unc-6* and *unc-40* mutants, the lack of AC invasion is not causing gross fate mis-specification (Table **3-2**).

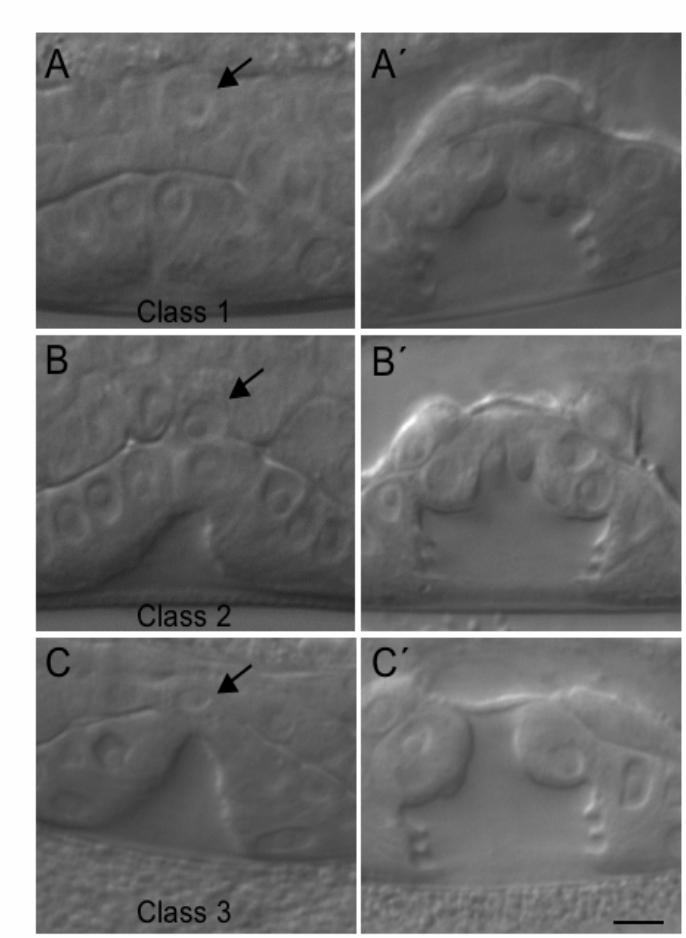

Figure **3-3**: AC behavior in L3 and early L4 is predictive of final vulva morphology outcome in mid L4. A) A phenotypic class 1 (see text) *unc-6(ev400)* null mutant. (A) The AC is displaced in the uterus and not adjacent to the vulva during early L4, and (A´) the morphology is abnormal in mid-L4. B) A phenotypic class 2 *unc-6(ev400)* null mutant. (B) The AC has broken down the basement membrane and sent processes towards the ventral lumen, but does not reside within vulF during early L4, and (B´) the morphology is abnormal in mid-L4. C) A phenotypic class 3 *unc-6(ev400)* null mutant. (C) The AC has fully invaded the vulva to reside within the vulF toroid by early L4, and (C´) the morphology is normal in mid-L4. Arrows indicate the AC. Scale bar: 5 μm.

Other AC invasion mutants exhibit a vulval morphology defect

The possible requirement of the AC for fate specification-independent steps in vulval morphogenesis is intriguing. To test the hypothesis that the physical presence of the AC in the center of the forming vulF toroid is necessary for proper vulval morphogenesis, I examined the vulval morphology of another mutant with defective AC invasion. As described in Chapter 1, *fos-1* acts cell-autonomously to promote basement-membrane breakdown by the AC to allow the AC to invade the vulva (Sherwood et al., 2005). In the *fos-1* mutant *ar105*, the basement membranes between the uterus and vulva remain intact and the AC rarely invades the vulva. I found that the mid-L4 vulval morphology of *fos-1(ar105)* mutants is abnormal and looks similar to *unc-6* and *unc-40* mutants (Fig **3-1**). To rule out a fate specification defect as the cause of this morphology defect, I also examined the fate specification markers for vulE, ZMP-1::GFP, and vulF, *lin-3*::GFP in the *fos-1* mutant. In all animals tested, these markers were expressed at the proper time and in the proper cells, indicating that the *fos-1* vulval morphology defect does not result from improperly specified vulval cells (Table **3-2**, Fig **3-3**). This correlates with previous data suggesting *fos-1* does not have gross fate specification defects (Sherwood et al., 2005). I conclude that the failure of the AC to invade the vulva and occupy the future luminal space of vulF results in defective morphogenesis of the dorsal vulva.

Polarity is normal in all AC invasion mutants

How the AC might affect the shape of the vulF toroid is unclear. It is possible that the physical presence of the AC is required to establish or maintain polarity in these late stages of morphogenesis to properly orient the apical domain of the vulF cell towards the lumen. To test this hypothesis, I examined whether polarity was perturbed in the AC invasion mutants.

I used a marker for the apical junction called AJM-1::GFP (Gidi Shemer, 2000; Sharma-Kishore et al., 1999). AJM-1::GFP not only indicates whether polarity is normal in the vulval cells, but it also provides a way to view the shape of the vulval cells. To look at this marker closely, I reconstructed a Z-series of confocal images to reveal the 3D structure of the vulva at mid-L4 stage. In wild-type animals, the apical edge of the vulE toroid appears as a small neck above which the vulF toroid has a wide junction with the uterus surrounding the large dorsal vulval lumen (Fig **3-4**). In *fos-1(ar105)*, AJM-1::GFP is expressed in all cells at the apical junction, suggesting polarity is normal (Fig **3-4**). However, the shape observed is quite different from wild type. vulF is clearly misshapen and comes to a point at the dorsal side instead of widening to form a proper connection with the uterus (Fig **3-4**). The pointed shape observed with AJM-1::GFP and the occluded dorsal vulva in the DIC images suggest that there is no lumen in the vulF toroid in *fos-1* mutants. The AJM-1::GFP expression pattern confirms my previous observation that the ventral vulva is normal. The variability in AC invasion in the *unc-6* mutants is reflected in variability in AJM-1::GFP reconstructions (not shown). However, *unc-6* and *unc-40* animals that lack AC invasion of the basement membrane completely (class 1)

appear very similar to *fos-1* (not shown). I conclude that vulval cell polarity is maintained in the absence of AC invasion.

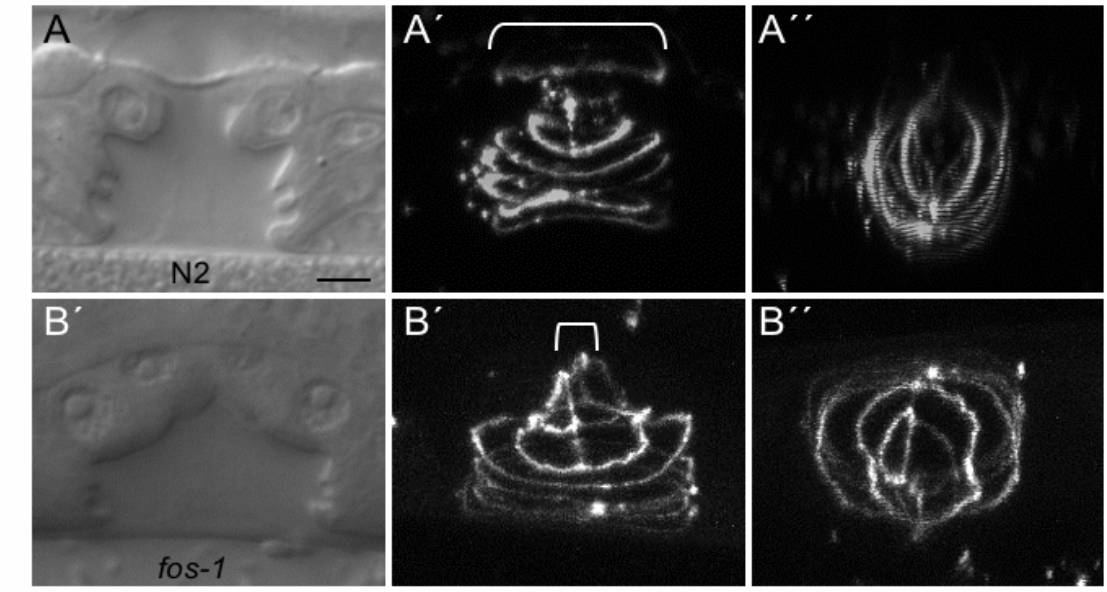

Figure **3-4**: Vulva cells with abnormal morphology are still properly polarized. DIC photomicrographs of mid-L4 vulvae of (A) N2 and (B) *fos-1(ar105)*. A´ and B´) lateral view and A´´ and B´´) dorsal view of 3D reconstructions of Z-series confocal images of AJM-1::GFP expression. Brackets indicate width of vulF at connection with uterus. Scale bar: 5 μm.

AC invasion mutants do not have a dorsal vulval lumen

My observations of dorsal vulval shape using AJM-1::GFP suggest that vulF lacks a lumen in AC invasion-defective mutants. However, as shown with *egl-26* mutants, AJM-1::GFP does not afford a complete view of the structure of the lumen. To

more directly test the hypothesis that AC invasion is required to establish a lumen in the vulF toroid, I used the GFP marker that is expressed at the membrane in vulE and vulF, TAT-2::GFP (Lyssenko et al., 2008), to visualize lumen formation in wild type and in *unc-40(e1430)* mutants that exhibit no AC invasion (class 1). I again reconstructed 3D images from a Z-series of confocal images. In wild type animals at late L3/ early L4, a clear pocket is developing in the vulF toroid at the site of AC invasion (Fig **3-5**). Since AC invasion occurs earlier than this stage, it seems that as the primary vulval cells divide and are pushed dorsally, they move up and around the AC which has already invaded. Once the primary vulval cells have finished their divisions and begun to fuse, a lumen has formed in the vulF toroid (Fig **3-5A**). As vulva development continues, the lumen widens, and once the AC has retreated to fuse with the uterine seam cell, a wide diamond-shaped lumen is clearly observed in vulF (Fig **3-5C**). In the *unc-40* mutant, the vulF cells are misshapen and appear disorganized during early L4 and no lumen has formed (Fig **3-5D**). At the mid-L4 stage, there is clearly no lumen in vulF (Fig **3-5E**). Based on my observations of few fate specification defects and completely normal polarity, as well as my visualizations of the differences in the shape of vulF between wild type and AC invasion mutants, I conclude that the physical presence of the AC is required to create a lumen in vulF.

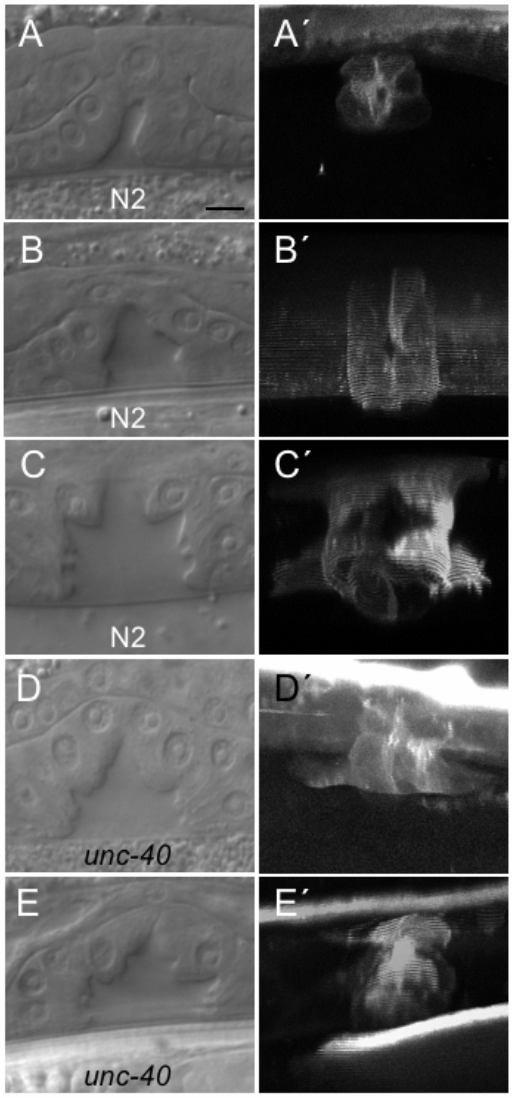

Figure **3-5**: The AC establishes the luminal space in vulF. Left panels are DIC photomicrographs of (A-C) wild type and (D,E) *unc-40(e1430)*. Right panels are 3D reconstructions of Z-series confocal images of TAT-2::GFP transgenic animals. (A) Late L3/early L4 N2. A pocket between vulF cells is visible showing the beginning of lumen formation. (B) Early L4 N2, the developing lumen is clearly visible. (C) Mid-L4 N2. A large lumen is visible. (D) Early L4 *unc-40(e1430).* No developing lumen is observed even at this early L4 stage. (E) Mid-L4 *unc-40(e1430)*. No lumen is present even at the mid-L4 stage when the lumen is the widest in wild type. Scale bar: 5µm.

Discussion

Incomplete invasion by the AC causes a morphology defect in the dorsal vulva

Lumen formation in the vulva requires proper specification of all vulval cell types during early stages of organogenesis as well as subsequent cell migrations, fusions and shape changes. I have characterized a set of mutants with similar morphology defects of the dorsal vulva at a post-specification stage of organogenesis. In *unc-6*, *unc-40* and *fos-1* mutants, the lumen in the most dorsal vulval cell, vulF, is absent. This defect does not arise from a fate specification defect. First, *fos-1* mutants have no vulva specification problems. Sherwood *et al.* demonstrated normal secondary versus primary vulval fate determination in *fos-1* mutants, and we have extended their analysis, demonstrating that differentiation of vulE and vulF within the primary lineage is normal. Second, while my data confirm a previous report that significant dorsal misplacement of the AC causes fate specification defects in vulE and vulF (Wang and Sternberg, 2000), such defects occur in only a minority of *unc-6* and *unc-40* mutants whereas the majority of the mutants have a dorsal morphology defect. Because these distinct mutations in UNC-6 or its receptor UNC-40 and the FOS-1 transcription factor share a defect in AC invasion of the vulva, I conclude that AC invasion of the dorsal vulva is critical to morphogenesis of vulF.

An issue to consider is whether these mutants are acting in a similar way in vulva morphogenesis because they each play a role within the vulva cells. UNC-40 is expressed early in the Pn.p cells, but whether or not expression persists in the VPCs during vulval development has not been reported (Chan et al., 1996). UNC-6 is

expressed in the vulva early in vulval development, it has also not been reported how long this expression persists (Asakura et al., 2007). It is also unclear what role vulval expression plays in AC migration as these studies focused on guidance of the hermaphrodite specific neuron. While *unc-6* and *unc-40* are clearly acting together to guide AC migration and thus mutation in each affects vulval morphogenesis in the same way, *fos-1* acts in a different aspect of AC invasion of the vulva. *fos-1* has two isoforms, *fos-1a* and *fos-1b*. Sherwood *et. al.* demonstrated that *fos-1a* is the functional isoform in AC invasion, it is not expressed in the vulva, and is required cell-autonomously in the AC for the AC to break down the basement membrane. In addition, the mutant used in this analysis, *ar105*, only affects the *fos-1a* transcript. While none of the experiments presented here can specifically rule out a potential role for these genes acting in the vulva specifically in vulva morphogenesis, it seems highly unlikely. Instead, their common effects on AC invasion of the vulva seem to dictate their effects on vulva morphogenesis.

Ablation experiments first demonstrated that the AC serves multiple functions during vulva development. Ablation of the AC prior to the onset or during the divisions of the vulval precursor cells or their progeny causes defects in the vulval lineage because of incomplete specification due to cell signaling defects (Kimble, 1981; Wang and Sternberg, 2000). Ablation at relatively late stages of vulva development, after all vulval divisions are complete, can also cause mis-specification of the vulE and vulF cell fate (Wang and Sternberg, 2000). Furthermore, ablation experiments at the beginning of the L4 stage have even hinted at late stage morphology defects (Kimble, 1981; Seydoux et al., 1993; Wang and Sternberg, 2000). Thus, an effect of the AC on late stage aspects of vulval morphogenesis is not unexpected. However, my work is the first to rigorously

examine a fate specification-independent role for the AC in vulva morphogenesis. I have clearly demonstrated that vulval morphogenesis goes awry, without perturbations in fate specification or polarity, when the AC does not occupy its expected position in the center of the developing vulF toroid. Thus, in addition to acting as a signaling center to coordinate development of the vulva and the ventral uterus (Kimble, 1981; Newman et al., 1996; Newman et al., 1995), the gonadal AC is required directly in the organogenesis process of the adjacent vulval tissue.

AC initiation of lumen formation provides a new model for studying initiation of epithelial tubulogenesis

The anchor cell breaks down the basement membranes and invades the developing vulva during L3. At this time, the vulval lumen has not developed. It appears that as the vulval cells continue to divide and as their migrations drive a dorsal invagination, the vulF precursor cells move around the AC that is already occupying the future dorsal luminal space at the midline of the developing vulva. The lumen of vulF begins as a pocket formed by the AC between the vulF precursors before they fuse to form the final vulF toroidal cell. This pocket enlarges as development continues, and eventually widens in an AC-independent manner after the AC withdraws by fusion with the uterine seam cell. In the absence of the AC, no pocket is formed in vulF and no lumen is seen, suggesting that this initial AC-occupied pocket is required for lumen initiation and that the vulF cell is not competent to initiate lumen formation, but can maintain and expand the lumen after the AC initiates its formation. In addition, my

analysis of *egl-26* suggests that even if the AC properly initiates the formation of the dorsal vulval lumen, other mechanisms are required for maintenance of the lumenal space. My work suggests the mechanisms of dorsal lumen initiation and dorsal lumen maintenance are two separate events.

Distinct mechanisms control dorsal and ventral vulval lumen formation

Only the portion of the vulva that forms the functional connection with the uterus is affected by the lack of AC invasion. The ventral vulva forms a normal lumen in the absence of AC invasion, suggesting separate mechanisms for lumen formation in the ventral and dorsal vulva. In the ventral vulva, the toroids develop around a large luminal space created by the migration-driven dorsal invagination of the vulval cells, an example of epithelial invagination. These toroids are formed as the widely separated cells of the anterior and the posterior secondary lineages join laterally, surrounding the luminal space (Dalpe et al., 2005; Sharma-Kishore et al., 1999). In contrast, the cells of the dorsal vulva arise from the single precursor of the primary lineage and are, thus, born in close proximity. The AC appears to ensure formation of the lumenal space in these more closely packed cells. I speculate that the AC-dependent mechanism evolved to ensure the functional connection between the vulval and uterine lumens.

UNC-6 plays multiple roles in egg-laying

Vulval morphology is critical to egg-laying function. The egg-laying phenotype of *unc-6* mutants was previously attributed to the AC migration defect (Hedgecock et al., 1990). Consistent with this conclusion, I have demonstrated that AC migration and subsequent invasion are required for dorsal lumen formation, which would affect egg-laying function. However, the egg-laying defect cannot be solely attributed to perturbations in AC migration or vulval morphology because the egg-laying defect is more penetrant than the vulval morphology defect. This is particularly striking when examining another allele of *unc-6, rh204*. In these mutants, 95% (n=103) of the animals have wild-type vulval morphology while only 45% (n=59) of these animals lay eggs. I speculate that effects of UNC-6 (Netrin) on axon guidance of the HSN (hermaphrodite specific neuron) (Adler et al., 2006; Asakura et al., 2007; Hedgecock et al., 1990), which is required for egg-laying function, also make a significant contribution to the egg-laying defect of *unc-6* mutants.

The *unc-6(ku243)* allele offers insight into separable functions of the UNC-6 protein domains. *unc-6(ku243*) is similar in penetrance to a null allele for the vulval morphology defect but is quantitatively and qualitatively less uncoordinated. The mutation in *ku243* results in a loss of only 80 amino acids at the C-terminus, in a domain called the C domain, suggesting that this domain is critical for AC migration and perhaps other similar cell migrations, but less important in axon guidance, which is required for proper movement. Previously, Lim *et. al.* demonstrated that the C domain may be only partially needed for AC migration. Moreover, the C domain is the domain which differs

most dramatically between species and the distinct functions of this domain are still under investigation (Barallobre et al., 2005; Lim and Wadsworth, 2002; Wang and Wadsworth, 2002). This newly identified allele may provide insight into the separate functions of UNC-6 (Netrin).

Materials and Methods

Maintenance and culture of *C. elegans* strains

Strains were grown under standard conditions at 20° C (Brenner, 1974). We used the following strains, alleles and transgenes: N2 wild-type; NW434 *urIs1*[*unc-6*::HA] (Wadsworth et al., 1996); PS4308 *syIs107*[*unc-119*(+) + *lin-3(delta-pes-10)::*GFP]; CB873 (eT1) (Rosenbluth and Baillie, 1981); IcEx982.11.2 (TAT-2::GFP) (Lyssenko et al., 2008); LG I: CB2261 *unc-40(e1430) dpy-5(e61)*; LG II: MH1385 *kuIs47* [AJM-1::GFP + *unc-119(+)*]; LG III: *unc-119(ed3)*; LG IV: PS3239 *dpy-20(e1282) syIs49*[*dpy-20*(+) + *zmp-1*::GFP]; LG V: GS384 *fos-1(ar105)/dpy-11(e224) unc-42(e270);* LG X: *unc-6(ev400, rh204, ku243).* Additional genetic information is available at www.wormbase.org.

Genetics

To create *fos-1*/eT1; eT1/+, we first mated N2 males to eT1 homozygous hermaphrodites. We selected male cross progeny and mated to *fos-1(ar105)/dpy-11 unc-42*. We selected hermaphrodite cross progeny and maintained the strain which had Pvl (indicating presence of *fos-1)*, wild type, Unc, and dead embryos/larvae progeny. To create *fos-1*/eT1; eT1/+ strains with GFP fate specification markers, we first mated N2 males into the GFP+ strains. We mated resulting GFP positive male cross progeny to eT1 homozygous hermaphrodites. We selected GFP+ males, which would be GFP/+; eT1/+; eT1/+, and mated these to *fos-1*/eT1; eT1/+ hermaphrodites. We then selected GFP+ hermaphrodites and selected the animals with progeny that exhibited the following: Pvl (indicating presence of *fos-1*), Unc and wild-type phenotypes and dead embryos/larvae.

To create *unc-6* and *unc-40* strains with GFP fate markers, we mated N2 males into GFP marker strains. We selected male cross progeny and mated to *unc-6* or *unc-40*. We then selected GFP+ hermaphrodites, allowed them to self, and selected GFP+ hermaphrodites that were Unc.

Microscopy

Images were acquired using a Zeiss LSM 510 confocal microscope or a Zeiss Axioplan 2. Z-series images were acquired every 0.5 μm and were re-constructed into 3D images using ImageJ (Abramoff, 2004). Some images were processed using

Photoshop.

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Chapter 4

Using the *C. elegans* vulva to understand initiation and maintenance of the lumen in tubulogenesis

My work has focused on understanding morphogenesis of the tubular organ, the vulva. I have studied two major aspects of vulva morphogenesis. First, I have expanded our understanding of *egl-26*, identifying its specific role in morphogenesis of the vulF cell. I hypothesize that EGL-26 acts in a non-cell autonomous manner. I have shown that it is a legitimate member of the NlpC/P60 superfamily of enzymes by demonstrating that the conserved catalytic residues are important for function *in vivo*. I have also shown that membrane localization of this protein is important for function. Close examination of the membrane, visualized with a membrane-localized GFP marker, has revealed that *egl-26* mutants have a dorsal vulval lumen, but it appears to collapse as morphogenesis progresses. These studies present the first evidence of an NlpC/P60 protein acting in metazoan development and detail the novel morphology defect observed in *egl-26* mutants.

Second, I have characterized the role of the gonadal anchor cell (AC) in vulva morphogenesis. In mutants that affect different aspects of AC invasion of the vulva, I have shown that the dorsal vulval lumen is not established. This absence of the AC in the dorsal vulva does not result in gross fate specification or polarity defects, and the evidence suggests that the presence of the AC is physically required in the vulva for dorsal lumen establishment. This study has also shown that the establishment of the

dorsal versus ventral vulval lumens are distinct events and that the AC is only required for dorsal lumen formation.

Taken together, my studies of *egl-26* and the requirement of the AC for lumen creation demonstrate that the mechanisms of lumen formation and lumen maintenance are separable and further our understanding of these events. In this chapter I will discuss the implications my work has on vulval morphogenesis in particular, and tubulogenesis in general.

Functions of the NlpC/P60 family in metazoans

EGL-26 is a member of the NlpC/P60 family of enzymes. Its closest mammalian homolog is lecithin retinol acyltransferase (LRAT), which is known to be a palmitoyltransferase. S-Palmitoylation is the post-translational addition of a palmitoyl fatty acid to a cysteine via a thioester linkage. Palmitoylation plays many roles, one of the most common is targeting a protein to the plasma membrane (Smotrys and Linder, 2004). It is an intriguing modification because in many cases it is reversible, allowing palmitoylation to be a mechanism of regulating protein function. While a few sequence motifs important for palmitoylation of individual proteins have been identified, a consensus sequence has not yet emerged (Smotrys and Linder, 2004). The only absolute requirement identified so far seems to be the presence of cysteine residues. This makes proteins that are palmitoylated difficult to identify based on their sequence alone.

The NlpC/P60 family is widely expressed, but little is known about the function of these proteins in metazoans, particularly within a developmental context. What is known about the biochemical mechanism of this family in eukaryotes comes mainly from studies of the mammalian protein LRAT, a palmitoyltransferase. LRAT is required for chromophore production in the retinal pigment epithelium and is responsible for palmitoylating all-trans-retinol to create all-trans-retinyl esters by accepting a palmitoyl group from vitamin A (Barry et al., 1989; MacDonald and Ong, 1988; Saari and Bredberg, 1989; Shi et al., 1993). LRAT acts via an ordered ping-pong mechanism (Shi et al., 1993). It accepts a palmitoyl group from its substrate and is transiently palmitoylated itself for a short time before binding to its second substrate and transferring the palmitoyl moiety. It has been implicated in regulating the membrane localization and function of another protein RPE65 (Xue et al., 2004), but that role has been disputed and instead it was suggested that LRAT affects RPE65 function simply by providing a substrate required for RPE65 function (Jin et al., 2007a).

Little is known about the role LRAT plays in tissues other than the eye. It is expressed in many tissues, at particularly high levels in the liver, lung, pancreas, intestine, testis, and of course the retinal pigment epithelium of mice and rats (MacDonald and Ong, 1988; Ong et al., 1988; Saari et al., 1993; Schmitt and Ong, 1993). However, in *Lrat* -/- mice, only defects in rod and cone function and occasional male infertility were observed despite very low levels of all-trans-retinyl esters in many tissues (Batten et al., 2004). In humans, a mutation in LRAT causes a form of retinal dystrophy, but no other phenotypes have been observed (Thompson et al., 2001). This mutation that causes retinal dystrophy corresponds with the *n481* allele of *egl-26.*

Recently, Lrat was implicated in zebrafish embryogenesis (Isken et al., 2007). Two *lrat* genes were identified, one involved in chromophore production in the retinal pigment epithelium (*lrata*), the other with broad expression in the developing embryo (*lratb*). However, Isken, ***et. al.*** showed that the effects seen on development caused by morpholino knockdown of *lratb* were due to a build up of retinoic acid and not some other effect of Lratb itself on development.

Another member of the LRAT sub-group of NlpC family is Hrasls3 (Anantharaman and Aravind, 2003). This protein was isolated as a tumor suppressor in Hras-transformed cell lines (Hajnal et al., 1994). Unlike LRAT, it has not been biochemically characterized. However, because it was identified as a tumor suppressor, it's implicated in playing a role in various cancers (Nazarenko et al., 2006; Nazarenko et al., 2007; Yanatatsaneejit et al., 2008), as well as adipocyte differentiation as a target of PPARγ (Hummasti and Tontonoz, 2008).

Palmitoylation and development

Several proteins that play fundamental roles in the development of all metazoans are palmitoylated (Miura and Treisman, 2006). Hedgehog (Hh) is palmitoylated by Skinny Hedgehog in *Drosophila*. This modification is implicated in Hh's ability to activate downstream targets (Chamoun et al., 2001). Another example is the Wnt pathway. Palmitoylation is shown to play a role in protein function in cell culture experiments as mutations in Wnts that prevent palmitoylation adversely effect function

(Willert et al., 2003). The defect seems to mainly involve loss of membrane targeting of un-palmitoylated Wnts, which results in a loss or reduction of function of the Wnts (Willert et al., 2003). In *C. elegans*, a loss-of-function phenotype is observed in the Wnt gene *egl-20* resulting from a mutation of the conserved cysteine shown to be palmitoylated in the cell culture studies (Maloof et al., 1999). In addition, proteins implicated as the acyltransferase enzymes in the Wnt pathway, like *porcupine* in *Drosphila* and *mom-1* in *C. elegans*, display phenotypes similar to those exhibited by their putative targets (Hofmann, 2000; Kadowaki et al., 1996; Rocheleau et al., 1997). Evidence from chick neural tube development indicates that palmitoylation of Wnts by Porc restricts its gradient thereby facilitating its function as a morphogen (Galli et al., 2007). The authors speculate this is because palmitoylation results in higher affinity for cell surface receptors. While these studies nicely demonstrate a role for palmitoylation in development, the acyltransferases in these pathways belong to a different family than *egl-26* (Anantharaman and Aravind, 2003; Hofmann, 2000). Also, *egl-26* mutants do not display phenotypes of any of these pathways, as would be expected were it functioning there.

The targets of *egl-26* are unknown. Yeast-two-hybrid experiments pulled out potential targets, but none of these seemed to function with *egl-26* in initial studies (Rita Sharma, Hongliu Sun, unpublished data). My data suggests that EGL-26 is acting as a palmitoyltransferase based on mutagenesis of the catalytic residues responsible for catalytic function in LRAT. Our lab has also investigated the proteins that share closest homology with RPE65 in *C. elegans*, but RNAi knockdown alone or in an *egl-26* mutant background showed no phenotype (Hongliu Sun, unpublished data). Rasika

Kalamegham investigated the role of retinol, the palmitoyl donor for LRAT, in *C. elegans*. There is no evidence that this crucial factor in mammalian development plays a role here, and her investigations using HPLC, visualization of autofluoresence and radio-labeling of fatty acids showed little or no detectable differences between wild type and *egl-26* mutant animals.

It is likely that the target of EGL-26 palmitoylation is a factor that acts non-cell autonomously based on the following evidence; EGL-26::GFP is expressed in vulE but the mutant phenotype is observed in vulF, palmitoylation plays a role in targeting proteins or other factors to the membrane, and EGL-26 itself is localized to and functions at the membrane. The identity of this target or targets is still unknown and would increase the understanding of the role of palmitoylation not only in vulva development, but also metazoan development as well. It also may elucidate a role for a currently unknown factor in lumen formation, either a factor that may interact with the extra-cellular matrix or a factor that changes the composition of the apical membrane of vulF.

Lumen formation in *C. elegans*

There are few known examples of proteins or factors important in lumen formation in *C. elegans*. A class of mutants called Excretory canal abnormal (Exc) exhibit defects in proper growth of the excretory canal lumen (Buechner et al., 1999). However, most of these mutants are defective in later stages of the elongation of the canal during larval development, not during initial lumen formation. In addition, mutation in

these genes results in cyst creation, or an increase in diameter of the lumen, as opposed to the phenotype of lumenal collapse or absence of lumen observed in *egl-26* and AC invasion mutants.

The most well-known example of defective lumen formation in vulva development is the Squashed vulva (Sqv) mutants. These mutants have a smaller vulval lumen than wild type animals, but are normal in cell proliferation, migration and vulva toroidal cell fusion (Herman et al., 1999). Unlike the mutants I have described where only the dorsal lumen collapses, in Sqv mutants the entire vulval lumen collapses. All of the eight Sqv mutants are members of the glycosaminoglycan biosynthesis pathway. Six of the eight members are involved in biosynthesis of heparan sulfate, the other two in synthesis or processing of chondroitin (Herman et al., 1999; Hwang and Horvitz, 2002; Hwang et al., 2003). In *Drosophila*, heparan sulfate influences the distribution of well-known signaling molecules in development, such as Wingless, Fibroblast Growth Factor and Hedgehog (Baeg and Perrimon, 2000). The first evidence of heparan sulfate regulating a signaling factor *C. elegans* was recently found (Gumienny et al., 2007), suggesting these extra-cellular components may have functions in *C. elegans* development that have not been discovered. It is interesting to speculate what chondroitin and heparan sulfate may be doing to expand the vulval lumen. One possibility that has been suggested is that chondroitin interacts with water, generating osmotic pressure and swelling of the lumenal space (Hwang et al., 2003). Another is that chondroitin proteoglycans cause a stiff extra-cellular coat on the secreting cells, in this case the vulva cells, and this helps shape the vulva tube (Podbilewicz, 2004). Perhaps *egl-26* modifies one or more of these extra-cellular matrix proteins to target it specifically

to the vulF cell membrane resulting in either swelling of the dorsal vulval lumen or a stiffening of the vulF apical membrane.

C. elegans vulva development as a model for tubulogenesis

The *C. elegans* vulva can be used as a model to study the basic mechanisms of tubulogenesis. I have shown that the gonadal anchor cell plays a role in lumen formation, but other key players have yet to be identified. As described above, the Sqv mutants offer an interesting avenue to study how the extra-cellular matrix plays an essential role in lumen formation. However, many fundamental questions remain about tubulogenesis that may be answered by studying the vulva.

Lumen creation is being studied in many systems. In the *Drosophila* trachea, for example, lumenal size is not dictated by number of cells and there seem to be distinct, developmentally controlled mechanisms for determining diameter and length (Beitel and Krasnow, 2000). In zebrafish vascular development, mutants that affect lumenal size by either causing a dilation or collapse of the lumen have been identified (Jin et al., 2007b), and in the *C. elegans* excretory canal, mutants that cause tube dilation have been identified, and in some cases molecularly characterized (Buechner, 2002). However, it is important to note that in all these examples, the lumen is initially formed and the

phenotypes seen are indicative of disruptions in later lumen development. These phenotypes are similar to what is seen in *egl-26* mutants in that a lumen is established, but it appears to collapse, preventing proper function. In contrast to the above examples, the phenotype observed in the AC invasion mutants indicates that the process of lumen formation is not even initiated. Examining the requirement of the AC for lumen formation may therefore lead to insights into lumen initiation as opposed to lumen maintenance or size regulation.

Another aspect of tubulogenesis involves fusion of previously formed tubes. Two organs may develop separately, but need to be connected in order to function. This occurs in mammalian esophageal and stomach development (Troy L. Spilde, 2002). These organs develop separately and the tubular esophagus grows toward the developing stomach and eventually they need to connect. In esophageal atresias, the stomach and esophagus do not meet and the individuals need corrective surgery, usually as infants, to rectify this defect (Brunner and Bokhoven, 2005). In other cases, different branches of tubes within the same organ need to fuse and form connections, as seen in the *Drosophila* trachea (Samakovlis et al., 1996). This type of coordinated development is difficult to study in complex systems because of the hundreds of cells and multiple tissues involved. The uterine and vulval connection in *C. elegans* is an ideal model since there are so few cells and the AC seems to be the only cell required to form this connection. In addition, these organs are not essential for viability, allowing for recovery of mutants involved in this process.

The requirement of the AC in forming the lumen of an adjacent tissue and then removing itself so a functional connection can be made is an interesting idea. It is

possible this type of coordination occurs in other, more complicated systems. How do two organs meet and connect? How do two fully-formed lumens fuse to form one continuous functioning lumen? The same barriers that exist between the uterus and vulva exist between tissues in other organisms, like basement membranes, for example. Perhaps an apoptotic event to remove cells between two lumens can occur to form a functional connection. Recent evidence from kidney development indicates that apoptosis of the common nephric duct allows ureter translocation which leads to formation of a connection with the urogenetal sinus epithelium, the primitive bladder structure (Batourina et al., 2005). While this study did not address the actual process of fusion of these two structures, it does highlight that apoptosis can act to reposition two organs to facilitate in their connection. An understanding of how these fundamental mechanisms work in *C. elegans* will lead to a better understanding of these processes in higher eukaryotes.

Conclusion

My work on *C. elegans* vulva development leads me to propose the following model of lumen formation and maintenance. I have shown that the AC is required for lumen formation of the dorsal vulval lumen by physically occupying the future lumenal space in vulF. When the AC is absent or has not fully penetrated between the vulF precursors, an abnormal vulva is observed. It is interesting to note that early in L4, it appears as though the AC is forming a pocket in the vulF cell, but that it does not completely penetrate through the ventral side of the cells to the vulE lumenal space.

Further, my observations suggest that the connection between the dorsal and ventral vulval lumens occurs after initial lumen formation. As vulva development continues, the AC withdraws and fuses with the uterine seam cell. It is at this stage when EGL-26 functions to maintain the lumenal structure in the dorsal vulva. In keeping with this hypothesis, we observe initiation of EGL-26::GFP expression at this stage in development. EGL-26 functions by plausibly modifying a secreted factor required in the extra-cellular matrix for lumenal structure, or a factor required to maintain rigidity of the vulF apical surface.

The obvious next step in understanding vulval morphogenesis is identifying the targets of EGL-26. Given that no *egl-26* alleles, even the likely null, are fully penetrant for the Egl defect, it suggests that *egl-26* may function in partnership with one or more proteins. A screen for suppressor or enhancer mutants may yield these putative co-conspirators. Another approach would be to find mutants with a similar phenotype to *egl-26* since mutations in the *egl-26* target would be expected to have a similar phenotype. Attempts to express EGL-26 in insect and mammalian cell culture to conduct biochemical assays have thus far failed, but could be attempted in other systems.

It would also be informative to examine other nematode species to see if the AC plays a similar role in vulva establishment. Examination of the genes involved in cell remodeling during morphogenesis has yet to be closely explored in *C. elegans*. For instance, why are the ventral vulval cells competent to remodel and form a lumenal structure while the dorsal vulval cells are not? Is this due to the migration of the cells alone? Or are there other factors involved? It would be interesting to examine the

membrane composition of the dorsal versus ventral vulval lumen to determine whether differential membrane compositions play a role.

Although many questions remain to be resolved, it cannot be disputed that the *C. elegans* vulva offers an elegantly simple *in vivo* model to understand the various complex processes underlying tubulogenesis.

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Honorable Mention Teaching Assistant Award, fall 2004

PLATFORM PRESENTATION

16th International *C. elegans* Meeting, University of California, Los Angeles, 2007.

POSTER PRESENTATIONS

Society for Developmental Biology Annual Meeting, Philadelphia, PA 2008.
American Society of Cell Biology Annual Meeting, Washington DC, 2007.
16th International *C. elegans* Meeting, University of California, Los Angeles, 2007.
The Graduate Exhibition, The Pennsylvania State University, 2007.
Mid-Atlantic Regional Society for Developmental Biology Meeting, Carnegie Institute, Baltimore, MD, 2006.
15th International *C. elegans* Meeting, University of California, Los Angeles, 2005.