Characterization of the *fhod*-2 gene
in *Caenorhabditis elegans*

by

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Abstract

Formins are a family of proteins responsible for a vast array of cellular functions such as cell shape, adhesion, cytokinesis and morphogenesis. Formin regulation of actin is a highly regulated process and is conserved from yeast to mammalian systems. Previous studies have shown that formins alone are necessary and sufficient for proper recruitment, polymerization and nucleation of actin filaments in the cell. The Chin-Sang lab is interested in studying development in C. elegans. In particular, one area of our research focuses on trying to understand different pathways and their related genes that ultimately determine cell shape changes and cell movements. This is an important area of research as many genes found in C. elegans have homologous counterparts in the human system. Studying these simple nematode worms may provide us insight into how the much more complex human body functions. Ultimately, research may provide clues or strategies for future treatments such as gene therapy. The focus of my research is on the fhod-2 gene. fhod-2 (Formin HOmology Domain) is a member of the formin family. Our lab has previously shown that this gene may be involved in early embryo development. Through characterization of a fhod-2 deletion mutant, I have been able to further elaborate on this work. Through the use of over-expressing lines, RNAi, protein expression and DIC microscopy I have identified defects caused by this mutation. fhod-2 is necessary for a variety of cellular functions both early in the embryo and later for proper development of the organism. FHOD-2 in situ antibody staining revealed expression in the pharynx and leading/seam cells, western blots of FHOD-2 over-expressing lines also detect higher amounts of FHOD-2 protein in embryos suggesting an
embryonic function. I have provided evidence that *fhod*-2 is a maternal effect lethal gene with defects seen in cytokinesis, gastrulation and elongation. DIC imaging of maternally rescued *fhod*-2 mutants reveal a plethora of defects affecting the head, gonad and excretory canal cell. Lastly through the use of florescence reporter constructs, *fhod*-2 mutants have neuronal defects displaying over-extension, early termination or branching of neurites.
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# List of Abbreviations

ADF – Actin-Depolymerizing Factor  
AMP - Ampicillin  
ARP – Actin Related Protein  
BLASTp – Basic Local Alignment Search Tool Program  
bnil – Yeast formin (Bud Neck Involved)  
bnr1p – Yeast formin (bnil related protein)  
CAR – Cytokinetic Actin Ring  
CC – Coiled-Coil region  
cDNA - Complementary DNA  
cdc12p – Yeast formin (Seventh Homolog of Septin)  
*C. elegans* - *Caenorhabditis elegans*  
CFB – Circumferance Actin Bundle  
CLIC – Chloride Intracellular Channels  
Cyk-1 – CytoKinese defect-1  
DAAM - Disheveled-Associated Activator of Morphogenesis  
DAD - Diaphanous Auto-regulatory Domain  
DAPI – 4’,6-diamidino-2-phenylindole  
DD – Dimerization Domain  
Dia - Diaphanous  
DID – Diaphanous Inhibitory Domain  
DIC - Differential Interference Contrast  
DiI - 1,1'-Dioctadecyl 3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (fluorescent carbocyanide dye)  
DRF - Diaphanous Related Formins  
DTC - Distal Tip Cells  
ECL - Enhanced ChemiLuminescence  
*E. coli* - Escherichia coli  
eT1 – UNCoordinated (also known as *unc*-36)  
exc – EXcretory Canal Abnormal  
FH - Formin Homology  
FHOD – Formin Homology Domain  
FITC – Fluorescein Isothiocyanate  
FMN – Formin  
FRL - Formin-Regulated gene in Leukocytes  
for3p – Yeast formin  
fus1p – Yeast formin  
GBD - GTPase Binding Domain  
GFP - Green Fluorescence Protein  
hmp – HuMPback  
HRP - HorseRadish Peroxidase  
IC – Worm Strains (Ian Chin-Sang Lab)  
INF - Inverted formin
L1 - Larval stage one
L2 – Larval stage two
L3 – Larval stage three
L4 - Larval stage four
let-502 – LEThal
mIs10 – myo-2::gfp marker
mRNA - Messenger ribonucleic acid
mel-11 – Maternal Effect Lethal
N2 - Wildtype
oIC – Oligos (Ian Chin-Sang Lab)
PCR - Polymerase Chain Reaction
PDZ – Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein
pIC – Plasmid (Ian Chin-Sang Lab)
PH - Pleckstrin Homology
quis – Queen’s University Integration allele
RFP - Red Fluorescence Protein
RNAi - Ribonucleic Acid Interference
ROCK – Rho-kinase
ruIs32 – pie-1 pormoter/histone H2B::GFP
SDS PAGE - Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
TET – Tetracycline
UNC – uncoordinated
UTR – Untranslated Region
WASP - Wiskott-Aldrich Syndrome Proteins
WT – Wildtype
Chapter 1

Introduction and Literature Review

*Caenorhabditis elegans* is a widely studied model organism. The transparent nature of *C. elegans* makes it an ideal model to follow development. Having the entire cell lineage mapped out allows one to easily trace the origin of cells and determine when and how they will be made. It also allows one to pinpoint when and where things go awry. Development in these worms are virtually invariant which provides an excellent model for one to study the role of certain genes at single cell resolution. Due to early lineage experiments, we know what happens during morphogenesis at a descriptive level but the molecular mechanisms underlying them are less understood. Under a variety of signaling pathways, cells must communicate with each other to migrate from their surroundings and reposition themselves for proper development of the organism. This may involve breaking old adherence junctions and forming new ones, adhering to new cells and remodeling of cell shape. Starting from a single cell, the organism needs to undergo a variety of cytoskeleton changes to become the familiar vermiform shape (figure 1). This process has been widely studied and mechanisms driving these events are well understood. The three major morphogenetic events that the *C. elegans* hypodermis must undergo are dorsal cell intercalation, enclosure of the ventral surface and elongation (figure 2). The hypodermis originates as six rows of cells on the dorsal surface of the embryo. During dorsal intercalation, the two innermost rows of cells interdigitate to form a single row of cells (Simske and Hardin 2001). For ventral enclosure, the two outermost rows of cells migrate over the surface of the embryo and meet at the ventral midline,
enclosing the embryo in a single sheet of cells (William-Masson et al, 1998, Malik and
Hardin 1997, Piekny and Mains 2003). Following enclosure, elongation is driven by the
force of contractions of the circumferentially oriented actin microfilament bundles
(CFB), leading to a four-fold increase in length (Priess and Hirsh 1996, Chin-Sang and
Chisholm 2000).
Figure 1: Wildtype embryonic development of *C. elegans*.

(A) One cell (egg and sperm nuclei fusion), (B) the start of cytokinesis, (C) two-cell stage, (D) four-cell stage, (E) cell proliferation, (F) gastrulation, (G) ventral enclosure, (H) embryo turning on it side for elongation, (I) comma stage, (J) two-fold stage, (K) three-fold stage and (L) newly hatched larva (L1). Still images taken from timelapse movie using Normarski DIC optics. Time indicated in hours.
Figure 2: Schematic diagram of the three major morphogenetic events in *C. elegans*.

(A) Dorsal cell intercalation: the two innermost rows of the hypodermis cells interdigitate to form a single row of cells. Occurs in three steps, top bracket shows 6 single row of cells, middle bracket shows the cells being to interdigitate and bottom bracket shows cell fusion. Green dots represent sites of actin polymerization (B) Ventral enclosure: the hypodermis migrates towards the ventral midline to enclose the embryo, driven by actin filament rich filopodia. (C) Elongation: Circumferential actin bundles in the hypodermis act to squeeze the embryo, driven by the force of contractions of the circumferentially oriented actin microfilament bundles (CFB). (D) Circumference actin filament bundle during elongation, contraction decreases the circumference of the animal. Dorsal hypodermis cells (pink), lateral hypodermis cells (yellow) and ventral hypodermis cells (red). Figure adapted from Chin-Sang and Chisholm 2002, Marston and Goldstein 2006.
The actin cytoskeleton is essential for these processes, for example, cells extend actin-rich filopodia as they elongate towards the ventral midline and are held together via adherence junctions which are also composed of actin. Embryos treated with actin polymerization inhibitors such as cytochalasin D show failure of dorsal intercalation and elongation is also blocked as cytochalasin D also disrupts CFBs (Marston and Goldstein 2006, Priess and Hirsh 1986). Actin also requires regulators, genetic screens have revealed more than 30 genes required for regulation of the actin cytoskeleton, when mutated these genes cause defects in elongation and cytokinesis defects. For example, mutations in the hmp-1 and hmp-2 genes in C. elegans cause ventral enclosure and elongation defects. The hmp (humpback) genes belong to a family of cadherin/catenin complexes that link cell adhesion to the actin cytoskeleton (Costa et al, 1998, Sutherland and Witke 1999). Other genes found in C. elegans work antagonistically with each other, let-502 (Rho-binding kinase) causes an elongation defect whereas mel-11 (myosin phosphatase targeting subunit) leads to hyper-elongation (Piekny et al, 2003, Simske and Hardin 2001,). Mutations in these genes lead to failure of cells and actin filaments attaching to adherens junctions and unregulated CFB contractions. Ultimately, proper morphogenesis involves a partnership between these gene products and actin. Studies point to actin as the central organizer of the cell and a vital component in the development and maintenance of cell shape, motility, stability and cellular transport (Ding et al, 2004). As actin is required for many diverse processes in the cell, deciphering its specific contribution to different cellular processes during development can be a challenge. Many morphogenetic movements observed in C. elegans are similar
to other organisms, for example ventral enclosure has many similarities to dorsal enclosure in *Drosophila* (Jacintoa and Baum 2003). Studies of actin and its regulators should provide a better understanding of how these components work together, to determine parallels between pathways and identify conserved functions among different organisms.

1.1 Actin Regulation

The role of actin in the cell is highly diverse. Actin plays a vital function in almost all cellular functions from yeast to mammalian cells and is widespread from the nucleus, cytoplasm and cytoskeleton. It is a life long player, its presence necessary from the very start of life. For example, actin is needed to regulate transcription and translation (Miralles and Visa 2006). Beyond that, actin is found in numerous cellular structures and is required for cell signaling. The basic mechanism of actin filament dynamics is not well understood; while there have been many identified factors that help control these dynamics, how they work together to modulate filament turnover is not well understood. G-Actin or gobular actin are monomeric units that are formed into F-Actin or filamentous actin via actin nucleators have been well studied and characterized. Actin filaments exist in a steady state and can be polymerized or depolymerized by the addition or loss of actin subunits. Filaments are polar structures with a fast growing (barbed) end and a slow growing end (pointed) end (Goode and Eck, 2007, Schonichen *et al*, 2005).

The balance between actin filament growth and shrinkage is very important for the function of the cell and probably differs between various structures. For example,
filapodia may require fast actin polymerization and depolymerization to provide efficient cell movement. Regulation of these structures may not be as tightly regulated as formation of the cytokinetic actin ring (CAR). CAR requires coordinated membrane remodeling of the cleavage plane for proper cell division (Castrillon and Wasserman 1994). It is clear that in the latter case, slow and accurate actin dynamics would be beneficial as consequences of disruption are much greater and can result in cell death. It is proposed that the presence of actin-depolymerization factor (ADF)/cofilin, a family of actin binding proteins that disassociates actin filaments, mediates the shortening of filaments (Michelot et al, 2007). In vitro studies show that single actin filaments rapidly grow and shorten at a steady rate maintaining constant filament length. ADF/cofilin plays a central role in filament length by trimming the end of the aged filament while growth continues on the barbed end (Michelot et al, 2007).

Past studies have revealed five types of actin nucleators, the Arp 2/3 complex, formins, Spire, Cordon bleu and leiomodin (Liu et al, 2008) (figure 3). Of these the Arp 2/3 complex and formins are the most researched and understood. It is named after the Arp2 and Arp3 subunits that form this 7 subunit complex. The Arp 2/3 complex is one of the most energy efficient actin nucleators. Studies reveal that it is a sedentary complex that creates new filament branches on pre-existing filaments upon activation by WASP family members (Sawa et al, 2003, Higgs and Pollard 2001, Pollard et al, 2000). Formins on the other hand are less efficient, they form a 2 subunit complex and nucleate denovo formation of linear unbranched actin filaments via actin dimer stabilization (Zigmond
2004, Goode and Eck, 2007). They move progressively with the elongating filament at the barbed end which prevents capping proteins from terminating filament growth and are ideal for generation of long actin filaments (Cooper and Pollard 1985). Spire is unique in that nucleation creates unbranched actin filaments through formation of a prenucleation complex containing up to four actin monomers (Kovar 2006, Quinlan et al, 2005).

Similar to the Arp2/3 complex, Spire is bound to the pointed end of the filament meaning that growth is limited due to capping proteins. Cordon bleu is believed to function in a similar manner to formins but as a trimer and is structurally different (Ahuja et al, 2007).

Lastly, leiomodin is a relatively recent discovery and not much is known about it to date. It is thought to nucleate linear actin filaments through the formation of a trimeric structure and is bound to the pointed end of actin filaments (Chereau et al, 2008).
Figure 3: Five types of actin nucleators.

A) Arp 2/3 complex named after the Arp2 and Arp3 subunits that form this 7 subunit complex, a sedentary complex that creates new filament branches on pre-existing filaments at 70° angles. B) Spire is unique in that nucleation creates unbranched actin filaments through formation of a prenucleation complex containing up to four actin monomers, it is bound to the pointed end of actin filaments. C) Formins form a 2 subunit complex and nucleate formation of linear unbranched actin filaments via actin dimer stabilization, they move progressively with the elongating filament at the barbed end which prevents capping proteins from terminating filament growth. D) Cordon bleu is believed to function in a similar manner to formins but is structurally different as a trimer. E) Leiomodin is a relatively recent discovery and not much is known about it to date, it is thought to nucleate linear actin filaments through the formation of a trimer and is bound to the pointed end of actin filaments. Pink circles represent actin subunits.

Figure adapted from Goley and Welch 2007
1.2 Formins

My focus is on the formin family of actin nucleators. Formins share two regions of sequence homology and were named after the observed phenotype of limb deformity first seen in rats when the *formin-1* gene is impaired (Woychik *et al*., 1998). Currently there are 7 groups of formins; Dia (diaphanous), DAAM (disheveled-associated activator of morphogenesis), FRL (formin-regulated gene in leukocytes), FHOD (formin homology domain-containing protein), INF (inverted formin), FMN (first original formins) and delphilin (Goode and Eck, 2007, Schonichen *et al*., 2005). Dia, DAAM and FRL are also known as the diaphanous related formins (DRF) as they share structural similarities outside of the FH1 and FH2 domain. The DRFs share common domain structures such as a GTPase binding domain (GBD), a diaphanous inhibitory domain (DID), a coiled-coil region (CC) and a diaphanous auto-regulatory domain (DAD) (Lu *et al*., 2007). Formins are an ubiquitous family of eukaryotic proteins that potently influence actin polymerization dynamics. They are large multi-domain proteins responsible for actin filament assembly and remodeling for a vast array cellular processes such as development of the cytoskeleton, epithelial guidance, cytokinesis, morphogenesis, establishing polarity and assembly and maintenance of adherens junctions (Homem and Peifer 2008, Waller and Alberts 2003). Formins also increase the rate and duration of filament elongation by preventing barbed end capping from capping proteins (Higgs 2005). There is also evidence that formins are regulators of microtubules and are involved in their stability (Dettenhofer *et al*., 2008, Kanaya *et al*., 2005). Members of the formin family share two unique and conserved regions known as formin homology 1 and
2 (FH1, FH2) domains (figure 4). Some formins also possess an additional region known as the FH3 domain, however it is less conserved and its function is not really known at this point in time. The FH1 domain is composed of a series of proline rich amino acids and recruits profilin actin complexes and increases the rate of filament elongation (Aspenstrom et al, 2006, Goode and Eck 2007). In the mouse formin mDia1, profilin has been shown to increase the rate of elongation 10 fold compared to unbound filaments (Romero et al, 2004). The FH1 domain ensures that formins can efficiently use the profilin bound actin as it is the most abundant form of monomeric actin available for polymerization in eukaryotic cells (Pollard 2007). Profilin is an actin binding protein involved in the restructuring of the actin cytoskeleton and controlled growth of actin filaments. Profilin also plays a regulatory role as profilin bound to actin cannot nucleate, so spontaneous nucleation is inhibited and the profiling-actin complex can only be added to the barbed end (Higgs 2005). The FH2 domain is a 400 amino acid sequence that is crucial for initiation of filament assembly associated with the fast growing barbed end which enables energetically favorable insertion of actin subunits and protects the end from capping proteins (Higgs 2005) (figure 5). The FH2 region alone is necessary and sufficient for actin nucleation (Zigmond 2004). While formins share a similar conserved FH2 domain responsible for initiating actin filament assembly and elongation, their roles in various animal systems vary and remains to be fully elucidated.
Figure 4: Domain structures of formins.

(a) Mammalian representatives from the seven groups of metazoan formins: Dia (diaphanous), FRL (formin-regulated gene in leukocytes), DAAM (disheveled-associated activator of morphogenesis), INF (inverted formin), delphilin, FHOD (formin homology domain-containing protein), and FMN (the original formins). Note that all formins have a conserved FH1 (yellow) and FH2 (green) domain, also depicted are less conserved putative diaphanous inhibitory domain (DID), coiled-coil region (CC), diaphanous autoregulatory domain (DAD) and PDZ domain (Psd-95/Dlg/Zo-1). (B) Expected domain structure of FHOD-2, a member of the DAAM subfamily of formin actin nucleators (shares 58% sequence identity). Figure adapted from Higgs 2005.
Figure 5: Mechanism by which formins nucleate actin polymerization.

(A) Normal filament elongation (pink circles are actin subunits). (B) Capping proteins (green rectangle) bounded to actin filament prevents elongation. (C) The two halves of the formin FH2 dimer are light blue and connected by flexible linkers (dark blue). The FH2 domain is responsible for initiation filament assembly by adding actin subunits onto the fast growing barbed end. The FH2 dimer protects the barbed end from capping proteins (green rectangle), allowing elongation even in its presence, but can act as a “leaky cap” to partially inhibit elongation. Figure adapted from Zigmond 2004.
1.3 Crystal structure is in consensus with formin function

Crystallographic analysis reveals that the FH2 domain is likely a dimer connected together by flexible connectors or linkers (figure 5). Analysis ranging from the human Daam1 formin to the yeast formin Bni1 suggests that this is characteristic of all FH2 domains (Lu et al, 2007). This dimer likely functions in a “stair-stepping” manner, as a fully bound dimer would sterically inhibit addition of actin subunits (Kovar 2006, Goode and Eck 2007, Higgs 2005). It is proposed that each half of the dimer makes alternating contacts with the actin filament. One half remains on the filament to prevent capping while the other repositions to allow for insertion of the profilin-actin complex between the FH2 domain and the barbed end of the filament (Xu et al, 2004). Although each half of the dimer is competent to interact with filament ends, the intact dimer is required for actin nucleation and processive capping.

Actin filament nucleation by formins remains to be fully elucidated but various studies have provided a consistent consensus on its function. Most formins such as the diaphanous related formins (DRF) appear to be autoinhibited via binding between its C terminal DAD domain and DID N-terminal sequences, and activated by Rho GTPase binding (figure 6) (Goode and Eck, 2007, Schonichen et al, 2005, Watanabe et al, 1999). Evidence supporting this theory comes from the fact that the FH2 domain alone is functional for actin nucleation and elongation and many truncated forms of formins with only the C-terminal, FH1 and FH2 regions behave as constitutively active (Evangelista et al, 1997, Kida et al, 2007, Lu et al, 2007). Biomimetic studies showed that formin Dia
(FH1 and FH2 region) coated beads along with a mixture of profilin and actin was sufficient for actin nucleation, and a higher concentration Dia correlated with increased nucleation rates (Michelot et al, 2007). DRF proteins lacking the DAD region also have substantially increased actin remodeling activity (Schonichen et al, 2005). However, their function may be slightly impaired when lacking its regulatory domain. Matusek et al (2006) found that expression of C-DAAM polymerized actin cables, but organization of these cables was not as highly ordered as in wildtype in Drosophila. It has also been proposed that Rho-GTP binding may act in a positive feedback manner (Habas et al, 2001). Once bound, it stabilizes the formin and acts to recruit additional formins. In addition to the inhibitory interactions between the C and N terminal, orientation of dimers may also be another mode of regulation. Yamashita et al (2007) found that the dimer protein structure tend to differ between species, in terms of number of helices, length of loops and flexibility between functional regions. For example, the human Daam1 formin is more compactly packed compared to the yeast Bni1 formin. The dimer halves are thought to fit together and block the binding site for actin filament activity. In human Daam1, the linker region is thought to stabilize the protein and mutations that disrupt the link between the dimer cause a 10-fold increase in formin activity (Lu et al, 2007). Similarly, stability of the FH2 dimer can also influence potency in nucleation activity. The Dia formins are highly potent nucleators and very low nM concentration are sufficient for nucleation while the FRLs are poor nucleators and require >100nM for similar effects (Harris et al, 2004, Li and Higgs 2003). This suggests that individual
formins can adapt to their unique roles with different modes of regulation and further research into the various elements of formin regulation is needed.
Figure 6: Schematic of the diaphanous related formin domain.

(A) Intramolecular interactions between the N-terminal DID and the C-terminal DAD keep the formin in an inhibited state. (B) GTP-Rho binding to the GBD causes activation and conformational change. The FH1 region recruits the profilin-actin complex and the FH2 region initiates actin filament assembly. GTPase-binding domain (GBD), diaphanous inhibitory domain (DID), dimerization domain (DD), coiled-coil domain (CC), formin homology 1 and 2 (FH1, FH2), diaphanous autoregulatory domain (DAD), profilin (P)

Figure adapted from Goode and Eck 2007.
1.4 Formin function differs between species

While all formins have the generalized structure and function of assembling actin, their role may or may not be similar. Sometimes formin function overlaps, as in the case of the yeast formin Bni1 and Bnr1p. These two formins have been found to be interchangeable and can be substituted for one another to promote actin filament assembly (Sagot et al, 2002). However, three formin isoforms of fission yeast Cdc12p, Fus1p and For3p nucleate actin for distinct and separate structures and functions and cannot be substituted for one another. Cdc12p is needed for the contractile ring formation and For3p for actin cable formation, whereas Fus1p is entirely different and is required for mating (Chang et al, 1997, Feierbach and Chang 2001, Petersen et al, 1998). Mammalian formins should be similar in terms of varying specificity in roles and degree of specialization. For example, the DAAM (Dishevelled-associated activator of morphogenesis) subfamily of formins has been implicated in Wnt/Fz planar cell polarity signaling pathway during gastrulation in Xenopus (Habas et al, 2001). They demonstrate that the Xenopus formin DAAM1 is required for RhoA activation. RhoA is a key regulator of cell polarity. The proposed signaling pathway is that the Wnt/Fz signal translocates Dsh to the plasma membrane which promotes the Dsh-DAAM1-RhoA complex. This activated RhoA activates a cytoskeleton remodeling via ROCK (Habas et al, 2001, Winter et al, 2001). ROCK (Rho-kinase) which is responsible for cell adhesion and motility. Loss of DAAM1 function studies reveal that RhoA activation is abolished, leading to morphological and gastrulation defects. The role of DAAM formin regulation functions via a similar regulatory pathway in zebrafish. DAAM1 is a coordinator of
endocytosis and cytoskeleton remodeling during notochord development (Kida et al., 2007). These authors found that DAAM1 mediates EphB molecules. Ephrins play a role in cell migration and adhesion. EphB endocytosis releases the adhesive properties of caudal cell surface of notochord cells, allowing elongation of these cells (Kida et al., 2007). F-Actin is also involved in aligning the direction of cell extension and support. Loss of DAAM1 results in abnormal shape of the notochord and loss of polarized cell movement.

In Drosophila, DAAM has been shown to be required for the regulation of the actin cytoskeleton in several tissues such as the tracheal system. In contrast to Xenopus and zebrafish, the role of DAAM in planar cell polarity is either absent or redundant (Matusek et al., 2006). DAAM activity is responsible for organising rows of parallel actin cables beneath the surface of the tracheal cells. They define the taenidial folds, which are thought to prevent collapse of the tracheal tubes and allows expansion and contraction of the tracheal cuticle. In wildtype animals these folds are highly ordered and form annular rings around the lumen of the tubes. Analyses of DAAM mutants reveal a haphazard order, shorter and thinner actin filaments that fail to develop around the circumference of the trachea. This results in the collapse and flattening of the trachea tubes. It was determined by (Matusek et al., 2006) that this is a problem in organisation as levels of f-actin in DAAM mutants remain constant. Over-expression of DAAM resulted in similar defects seen in mutants that lack DAAM. It is also interesting to note that this study also showed that DAAM loss of function mutants can also be lethal.
The *C. elegans* FHOD-2 protein closely resembles DAAM1 and is also a member of the DAAM subfamily of formin actin nucleators (figure 7). Previous characterization of the *fhod*-2 mutant phenotype was limited due to the fact that a mutant could not be isolated. We have since maintained a balanced *fhod*-2 deletion line with the *mIs10* GFP marked balancer chromosome (*mIs10/fhod*-2). There is no published role about the *fhod*-2 gene and its role in the cell, therefore its function and their role on various cellular processes remains to be fully elucidated. I will attempt to shed some light on this gene by characterizing the *fhod*-2 mutant phenotype using DIC microscopy imaging, antibody staining, and creating over-expressing lines. To provide evidence that the phenotypes that I observe are actually due to the *fhod*-2 deletion, I used a reverse genetics approach as well as *fhod*-2 over-expressing lines. Using RNAi to knock out the *fhod*-2 gene I showed that *fhod*-2 RNAi resulted in the same phenotypes seen in the mutant. By using a *fhod*-2 genomic construct I showed that the *fhod*-2 mutant defects could be partially rescued.
Figure 7: Schematic diagram of the *fhod-2* gene.

*fhod-2* is located on chromosome 5 and contains 7 exons, with a transcript length of 6443bp. The coding region is 2332bp long and translates into an 813aa long protein. The *fhod-2* mutant used in this study is *fhod-2(tm2133)* which has most of exon 3 deleted. This deletion region corresponds to the FH2 domain and most likely represents a null allele.
Chapter 2
Experimental Procedures and Results

2.1.1 Strains

All strains were grown and maintained at 20°C under standard conditions (Brenner, 1974). *fhod-2 (tm2133)* strains were tested at 15°C and 25°C with no discernable difference, indicating that this mutant is not temperature sensitive. Markers used in this work are: *ruIs32* (pie-1 promoter/histone H2B::GFP), GFP (green fluorescent protein), RFP (red fluorescent protein), *odr-1* (expressed in chemosensory neurons), *mec-4* (expressed in the 6 touch neurons), *sax-3* (expressed in all neurons, some hypodermal and muscle cells) and *OD70* (*unc-119 (ed3) III; itIs44 V pie-1p-mCherry::PH*). The only allele used in this project was *fhod-2 (tm2133)*. The Bristol N2 strain was the wildtype, all other strains used were created through crosses at the L4 stage. Basic strains were obtained from the *C.elegans* Genetics Center (CGC) and the Mitani group (Japan).

Strains used and created (described in detail below) are: wildtype (N2), *eT1/fhod-2*, *mIs10/fhod-2* (original line, IC638), *mIs10/fhod-2* (recombinant line, IC710), *mIs10/fhod-2* (outcross line, IC766), 9.7kb full length rescue line (IC826), 2 piece rescue line (IC857, quEx232), *mIs10/fhod-2; ruIs32* (IC823), *mIs10/fhod-2; bnIs1* (IC856), *mIs10/fhod-2 pfhod-2::gfp* (IC828, quEx234), *mIs10/fhod-2 pmec-4::rpf* (IC858, quEx233), *mIs10/fhod-2 psax-3::gfp* (IC860, quEx235), *mIs10/fhod-2 odr-1::rpf* (IC843), wildtype over-expressing *fhod-2* (IC861, quEx236), wildtype over-expressing *fhod-2* (IC861, quEx220), *mIs10/fhod-2* rescue line (IC827, quEx221), *mIs10/fhod-2* rescue line (IC862, quEx237), *mIs10/fhod-2* rescue line (IC863, quEx238), *mIs10/fhod-2* rescue line (IC828),
wildtype \textit{pfhod-2::gfp} (IC822), wildtype \textit{pmec-4::rfp} (IC83) and wildtype \textit{psax-3::gfp} (IC699).

2.1.2 Microinjection

To generate over-expressing and rescue lines the full length PCR product of the \textit{fhod-2} gene was amplified from wildtype genomic DNA and injected into \textit{mIs10/fhod-2} mutants. 2 constructs were made, a full length 9.7kb (oIC 257 Forward 5’CCTAAGCCTTTGGGTATTAATGTAATATGAAATTTAACTTTTT-3’, Reverse oIC 260 5’-ATTTGCCTGTTGTGGTCTTCTATTTATCGTCCCAT-3’ and oIC259 Forward 5’AACTGTGCCATCTACCGCAAACACTG-3’, oIC 427 Reverse 5’-CGACCGTTATTGCTGATGCCATCTACCGCAAACACTG-3’). These 2 fragments were then stitched together using oIC257 and oIC427) fragment. An 8.7kb (oIC 606 Forward 5’-CTTATCAGCGAAATGTACAGTATCGGCTGTAGAG-3’, oIC607 Reverse5’CAGTTGGTGATACGAACTATCAATTGATAAGAGA-3’) fragment that features a larger area of the promoter region (5kb) was also created. Two rescue lines were originally created, one featuring the 9.7kb piece alone and another with both the 9.7 and 8.7kb piece which were co-injected and allowed to recombine into a single extrachromosomal array in the animal. Injections were initially done at 30ng/µL and then at various other concentrations to determine the optimal concentration that will rescue the mutant phenotypes (1:500, 1:5000, 1:10000 and 1:25000). The transgenics that carried the extrachromosomal array were selected via a visible \textit{odr-1} RFP marker (30ng/µL) and maintained at 20°C.
2.1.3 Embryonic lethality/sterility

To determine lethality and sterility, I selected single mis10/fhod-2 hermaphrodites at the L4 stage and allowed them to mature and lay eggs at 20°C. Parents were transferred to a fresh plate every 24 hours until egg laying was arrested. Eggs were scored dead if they remained unhatched after 24 hours after removal of parents. To determine if the dead embryos can be attributed to the fhod-2 mutation they were checked for GFP fluorescence and via PCR. To determine if an animal was sterile, single L4 hermaphrodites were picked onto plates checked everyday for progeny until they died.

2.1.4 Microscopy and timelapse

Specimens were observed with a Zeiss Axioplan 2 microscope with Axiocam and Axiovision software. All microscopy unless otherwise stated was done on live animals mounted on 2% agarose pads anesthetized with 0.2% tricaine and 0.02% tetramisole. Animals were observed under the 40x and 63x objectives. To extract gonads, adult worms were picked onto a drop of M9 on a coverslip and cut at the pharynx or tail using a 20Gb1 (Precision Guide) needle to release the gonad. The coverslip was then inverted onto a slide with a 2% agarose pad. For timelapse movies, gravid adults were picked onto a watchplate and cut in half using a 20 Gb1 (Precision Guide) needle in a 10ul drop of M9 minimal buffer. Embryos were then transferred by mouth pipette to a 2% agarose gel pad set on a slide, covered with a coverslip and sealed with immersion oil (Zeiss Type B). Embryos were filmed at 63x objective. 8 planes at 2 um Z sections were taken every 1 minute for 8hrs. When GFP florescence was used images were taken every 5 minutes. All recordings were done at room temperature. This technique was tested with N2.
wildtype animals to ensure that the procedure did not hinder the natural growth of the animal. For the thrashing assay, worms were picked onto a drop of M9 minimal buffer set on a glass plate. They were filmed using a digital camera for microscope (DCM510) attached to a Leica MZ6 microscope. Worms were filmed for 5 minutes using ScopePhoto software and scored as moving or not moving (considered not moving if it was completely still after 5 minutes) after that time period.

2.1.5 Immunohistochemistry

Antibody Staining: Mixed stage and embryo staining was done using the freeze-thaw method and fixed with a 10% paraformaldehyde solution as described in (Chin-Sang et al, 1999). Mouse monoclonal MH27 (adherens junctions), chicken anti-GFP, and rabbit anti-FHOD-2 primary antibodies were all used as a primary. The fhod-2 antibody was previously made by Jadine Paw and a description of it can be found in (Paw 2005). I took this antibody and purified it using the Protien A IgG Purificaton Kit (Pierce) and against FHOD-2 protein (pIC244) to increase specificity. Texas Red conjugated goat anti-mouse, Texas Red conjugated goat anti-rabbit, FITC conjugated goat anti-rabbit, FITC conjugated goat anti-chicken were used as a secondary (Jackson ImmunoResearch Labs, Inc). Fluorescent secondary antibodies (1mg/ml) were used at a 1:500 dilution. Specimens were treated with Prolong Gold anti-fade (Invitrogen) before viewing.

Western Blotting: Embryo and mixed stage preps were lysed with 2X sample buffer + DTT. Samples were run on 10% SDS PAGE protein gels, western blotted and probed with anti-FHOD-2 antibodies (1:20 dilution) and HRP anti-rabbit secondary
antibody (1:2000 dilution). Depending on volume of worms 1-3 plates were used for each sample.

2.1.6 RNAi:

A plasmid (pIC333, isolated through a yeast-two-hybrid screen by Jadine Paw) containing a 1kb fragment encoding most of the FH2 region was digested with xhoI and purified by gel extraction (Promega SV Gel and PCR Clean Up System). The fhod-2 insert was ligated into the L4440 vector cut with xhoI and the resulting plasmid (pIC465) was transformed into the E. coli strain HT115 cells on an AMP TET plate and used for feeding.

2.2 Results

2.2.1 fhod-2 (tm2133) is a maternal effect lethal gene

My results suggest that fhod-2 is a maternal effect lethal or sterile mutation, hence it is impossible to maintain the strain in a homozygous state. Initially this strain was maintained as a balanced eT1/fhod-2 strain, as both homozygous eT1 and fhod-2 are lethal. This was problematic as it not possible to differentiate and score the phenotype of the fhod-2 mutant animals without PCR verification. One solution was to cross in the mIs10 (GFP balancer) animals into the eT1/fhod-2 strain. The rationale behind this is that it would be easier to maintain the strain and indentify mutants; mutants could easily be identified by the lack of GFP expression. I mentioned previously that fhod-2 mutants are lethal; however, I have found that it is possible for a small percentage of these homozygous mutants to survive. These survivors are known as escapers. While rare, it is
thought that they are able to avoid death by virtue of their mothers, a phenomena known as maternal effect. Maternal effect is the process whereby the mother makes a contribution, such as mRNA or protein to the zygote and the genotype of the mother is reflected in the phenotype of its offspring. A maternal gene product is passed on to the offspring which itself does not have the gene (can also be described as M+Z- meaning maternal copy present while missing in zygote). Maternal effect is important in early embryonic development as the maternal products can control the developmental program until embryonic genome activation takes place or compensate for the lack of gene expression in the offspring (Bernardo 1996 and Tong et al, 2000). This may be able to account for the small percentage of fhod-2 homozygous worms seen surviving. Further evidence supporting the role of maternal effect is that the surviving fhod-2 animals are sickly and do not go on to produce a viable next generation. However, these escapers have afforded us the opportunity to characterize the fhod-2 phenotype in adult worms.

We expected a certain number of fhod-2 mutant progeny from the rationale mentioned above, but during the course of maintaining the mIs10/fhod-2 strain an inexplicably high number of mutant progeny observed on the plates (percentage of fhod-2 mutant progeny increased significantly from 13.8% to 38.1%, P<0.005). It was hypothesized that perhaps the lethality originally associated with fhod-2 had now recombined off. In addition, during the course of maintaining the strain the mIs10 marker acquired a lethal mutation (percentage of mIs10 progeny significantly decreased from 62.7% to 44.7%, P<0.005). Supporting this notion was the fact that homozygous mIs10
animals could no longer be isolated. PCR testing of the dead embryos revealed that they were a combination of both homozygous mutants and wildtype or \textit{mIs10/mIs10}.

Outcrossing with a new \textit{mIs10} allele should remove the lethal mutation. Indeed this was the case, we eliminated this lethal mutation and saw a decrease overall embryonic lethality (17.7\% to 4.8\%, P<0.005) (figure 9). After outcrossing the lethality significantly decreased and ratios of \textit{mIs10} to \textit{fhod-2} progeny were at expected ratios of 75\% \textit{GFP} progeny (a combination of 50\% \textit{mIs10/fhod-2}, and 25\% \textit{mIs10/mIs10} as they both have a similar appearance) and 25\% \textit{fhod-2/fhod-2}. The low incidence of embryonic lethality (4.8\%) suggests that the majority of \textit{fhod-2} mutants can be maternally rescued.

The original \textit{mIs10/fhod-2} line with the \textit{fhod-2} linked lethal is named IC638, the recombined \textit{mIs10/fhod-2} line with the \textit{mIs10} mutated lethal is named IC710 and the outcrossed \textit{mIs10/fhod-2} line is named IC766. Most of the analyses hereon end will be using the IC710 line. The rational behind it is that maintaining the mutation is relatively easy and less likely to be lost as (since both \textit{mIs10/mIs10} and \textit{fhod-2/fhod-2} are lethal) all animals picked should be balanced \textit{mIs10/fhod-2}. Note that the linked lethal X gene can recombine back onto the \textit{fhod-2} chromosome but we can detect this as those \textit{mIs10/fhod-2} animals segregate a very small percentage of \textit{fhod-2} homozygous progeny (i.e. non \textit{GFP} animals).
Figure 8: Schematic diagram of the proposed mls10/fhod-2 chromosome.

(A) Original mls10/fhod-2 strain (IC638) featured fhod-2 which is lethal and an additional linked lethal gene X. This “double” lethality contributes to an increased percentage of fhod-2 deaths.

(B) Recombination event leads the lethal gene x to cross over, fhod-2 loses the linked lethal. Over time, mls10 becomes mutated and homozygous mls10 animals are now lethal (IC710). No discernible difference in overall lethality rate, but increase in fhod-2 homozygous progeny and decrease in mls10 homozygous progeny observed support this theory.

(C) mls10 linked to lethal gene X allele was replaced with a wildtype mls10 allele by outcrossing. Lethality rates decrease as linked lethal gene eliminated, but fhod-2 itself is still lethal (IC766). Bracket show expected ratio of progeny, WT denotes wildtype. Note the linked lethals can be on either side of fhod-2 or mls10, but shown on the right for illustrative purposes.
Figure 9: Graph showing percentage of total embryonic lethality of *fhod-2* mutants.

IC638 is original *mIs10/fhod-2* with *fhod-2* linked lethal gene X with 23.5% lethality (N=782, SEM = 3.8). IC710 (recombination and crossing over, now *mIs10* is linked with lethal gene X) strain showed 17.7% lethality (N = 1368, SEM = 4.85 Note: not significant difference) but had a decrease in *mIs10* homozygous progeny (62.7% to 44.7%), suggesting that now *mIs10* has linked lethal gene X. IC766 (outcrossed with new *mIs10* allele, linked lethal gene X removed) strain show lethality drops to 4.8% (N=213, SEM = 2.6) and was able to restore percentage of *mIs10* progeny back to expected wildtype levels. Error bars indicate the SEM and significant differences between strains are indicated above bars **P<0.01 and ***P<0.001.
A large part of my project involves using DIC imaging to observe defects that may be due to the \textit{fhod-2} deletion. I have provided a simplified cartoon diagram of \textit{C. elegans} (figure 10) that will help to orient the reader and highlight various anatomical features that I will be referring to in this thesis. \textit{fhod-2} is a gene predicted to act within the machinery that is responsible for recruitment and proper organization of actin, as a result, it should not be too surprising that the consequences of the mutation are widespread. I have used an older convention to differentiate between progeny from wildtype or mutant mothers. The M and Z refer to the mother and zygote respectively. The + or – indicates if the gene is present or lacking. For example, \textit{M+Z-} means that the mother had a wildtype copy while the zygote does not and \textit{M-Z-} means that both the mother and zygote did not have a wildtype copy of the \textit{fhod-2} gene. I have summarized the FHOD-2 expression pattern and characterized the main phenotypes below.
Figure 10: Simplified cartoon depiction of *Caenorhabditis elegans*.

Side view, from left to right, anterior (head) and posterior (tail) of worm. Green represents the pharynx, red represents the H shaped excretory canal cell which traverses the entire length of the worm. From this view you can only see part of the canal cell (solid line) and behind the gonad is the other part (dashed line). Blue represents the gonad, black triangle represents the vulva. I will be referring to the excretory canal cell and gonad in later sections.
2.2.2 fhod-2 (tm2133) mutants display defects in gastrulation and elongation

fhod-2 seems to play a role in embryonic development as embryonic lethality is observed in the mutants. To better understand how and at what stage these embryos are dying, I used time lapse video imaging of fhod-2 embryos. In a balanced mIs10/fhod-2 mutant, one would expect 25% or a quarter of these animals to be dead as fhod-2 homozygous animals are lethal. When embryos are filmed, it is done blind as I do not know their genotype at this stage. Due to chance it is likely that the sample size should include fhod-2 mutants for observation. In the timelapse videos, embryos were scored as heterozygous or wildtype if the GFP marker is present and as homozygous fhod-2 (M+Z) in the absence of GFP. As stated previously fhod-2 homozygous animals are usually lethal, but can be maternally rescued. Animals that die early in the videos were assumed to have been fhod-2 homozygous. From a sample size of N=38, 12 embryos or roughly 32% were homozygous fhod-2. 10 of these animals died from either a gastrulation or elongation defect, the two survivors are known as the escapers and probably survived due to the maternal contribution of wildtype fhod-2 gene products (i.e. maternal rescue). A normal C. elegans embryo takes about 12 hours to develop from a single cell into the universally recognized worm shaped larva. At approximately hours 2 and 7, the embryo undergoes gastrulation and elongation respectively (figure 11). Gastrulation requires that the surface cells invaginate the once simple ball of cells into a multilayer organism. Organ formation also occurs in this step. Elongation requires that the cells contract and change shape so the ball of cells is squeezed into a worm–like shape. Both these processes have been found to be abnormal in fhod-2 mutants.
**Figure 11:** *fhod-2 (M+Z-)* mutants are defective in gastrulation and elongation.

Top panels show proper wildtype development during gastrulation (A) and elongation (2-fold stage) (B) of wildtype animal. Bottom panel shows *fhod-2* mutants at similar stage of development (C,D). DIC images taken from timelapse experiment at 63X magnification. Cells in the mutant appear to have trouble dividing and elongation. They never develop further then the comma stage, this occurs in approximately 13-15% of mutants.
2.2.3 *fhod-2* (*tm2133*) mutants display early cell division defects

Although some of the *fhod-2*(M+Z-) animals grow up to adulthood, the vast majority are sterile. Suggesting that *fhod-2* may be involved in germline development. However some rare animals do lay eggs but these *fhod-2*(M-Z-) embryos die 100% of the time showing that *fhod-2* indeed is a maternal effect gene. These embryos die at an early stage and never make it past the comma stage of development. I showed that these *fhod-2* (M-Z-) embryos die within the first few cell divisions and have defects in cytokinesis, leading to cells with multiple nuclei (figure 12). Actin microfilaments have been shown to localize at the leading edge of the cleavage furrow (Swan *et al*, 1998). Defects in actin organization can lead to shortened actin filaments that fail to properly adhere leading to the failure of complete cellular division. Cell polarity is also necessary for cells to ingress. Cell polarity regulates the integrity and future orientation of cells and specific cellular cues are needed to form the polar ends of the embryo. Actin myosin becomes enriched at the apical surfaces which require polarities to distinguish between surfaces (Rohrschneider and Nance 2009). A failure in the polarization of the cell could cause inappropriate division of cellular content leading to the multinuclei phenotype.
Figure 12: *fhod-2*(M-Z-) mutants are defective in cytokinesis.

Top panel shows wildtype embryo (A) at 2-cell stage along with its corresponding GFP picture (B). Bottom panel (C,D) shows *fhod-2* mutant (M-Z-) with cytokinesis defect with multiple nuclei. *ruIs32* (*pie-1* promoter/histone H2B::GFP) marker was used to visualize DNA/nuclei. *fhod-2*(M-Z-) mutants die 100% of the time with a cytokinesis defect.
2.2.4 fhod-2 (tm2133) mutants have gonad morphology defects

The U-shape of the gonad is dictated by the migration of two leader cells, the distal tip cells (DTCs) as the worm grows. The DTCs originate from the ventral side of the 4 cell gonad primordium, it starts to migrate away then it turns and migrates to the dorsal side, followed by a second turn and migration towards the middle of the body (figure 13). Of the fhod-2(M+Z-) escapers the most apparent phenotype is an abnormal gonad morphology. Also, it is evident that the germline cells of the gonad fail to follow a proper sequence of development and most are necrotic (figure 14). As the nuclei in the distal tip of the gonad migrate to the proximal end, the nuclei should mature into oocytes. However, most of the nuclei in fhod-2 mutants are necrotic and oocyte formation is rare. Using both DAPI staining and the histone GFP marker (ruIs32), I show that while the DNA is present, it is improperly organized (figure 15). This may help to account for the high level of sterility observed in mutants. The role of actin inside the nucleus was once widely disputed, however many studies now support the link between actin and the regulation of chromatin structure. It is suggested that actin and the Arp formin are associated with chromatin remodeling complexes and histone modifying factors (Miralles and Visa 2006, Percipalle 2006, Percipalle and Visa 2006), the fact that the organization of DNA is impaired in mutants supports this theory. fhod-2 may be required for actin to properly coil the DNA and for proper cell division. Improper organization and development of the gonad may help to explain why mutants are usually sterile or produce embryos that are inviable. All M-Z- embryos are 100% lethal. There are also instances of somatic gonad migration defects leading to abnormal shape “blobby” and migration of
the gonad sheath. This was easily visualized using the \textit{itIs44 (pie-1p-m:cherry::PH)} marker that highlights the gonad membrane (figure 16).
Figure 13: Gonad migration in *C. elegans*.

Distal tip cells (DTCs, dark blue circles) are descendents of the Z1/Z4 lineage in the gonad primordium at L1. They begin to move away at L2 and turn towards the dorsal surface in L3 and another turn in L4 redirects the cells towards the center of the worm. Dashed lines represent gonad arm and path of DTC migration. Figure adapted from Meighan and Schwarzbauer (2007).
Figure 14: *fhod-2* mutant animals have necrotic nuclei.

(A) Wildtype worm showing half of gonad arm with proper nuclei shape and size, developing into oocytes. (B, C) *fhod-2* mutants show enlarged, necrotic nuclei that fail to mature into oocytes, resulting in mutant sterility. Gonads dissected out from adult worms.
Figure 15: *fhod-2*(M+Z-) mutants have unorganized DNA.

Top panel shows wildtype gonads with *ruIs32* (*pie-1* promoter/histone H2B::GFP) marker (green) and DAPI (blue) to visualize nuclei/DNA. Bottom panel shows *fhod-2* mutant (M+Z-) gonads. While level of DNA remains constant, the *ruIs32* marker reveals improper organization in *fhod-2* mutants. This may help to explain why the vast majority of *fhod-2* mutants (66%) are sterile. Mutants that do lay eggs are 100% letha.
**Figure 16:** *fhod-2* mutant animals have abnormal gonad migration.

Top panels are wildtype and bottom images are the same strain treated after *fhod-2* RNAi. In panels A,D red dash-line in cartoon shows migration defect of the distal tip. Wildtype gonad arm should resemble an U shape (B), in the mutant the distal tip travels opposite direction (E). Panels C,F shows the same images with an *itIs44* (pie-1p-m:cherry::PH) marker highlighting the membrane of the gonad. Posterior half of the gonad arm is shown.
2.2.5 *fhod-2 (tm2133)* mutants display excretory canal cell defects

Previous work showed that a *fhod-2::gfp* promoter could drive expression in the excretory canal cell (Chin-Sang Lab unpublished results). Thus, we wanted to examine if this excretory canal cell was affected in *fhod-2* mutants. The excretory canal cell is situated just under the hypodermis and is an H-shaped cell in *C. elegans* that runs the entire length of the organism. Formation of the excretory canal cell begins during embryogenesis, this cell must continue to spatially and temporally regulate its lumen diameter throughout its entire life (Buechner 2002). Mutations in the genes that regulate this cell can cause defects in length, lumen diameter and location of tube within the organism. Its function is osmoregulation via excreting saline fluid to maintain the animal’s salt balance, similar to a human kidney. It is an example of a tubal structure that may require actin for proper formation. Actin is necessary to help keep the lumen from collapsing onto itself. DIC imaging reveals defects ranging from early termination to variability in the widths of the lumen in the *fhod-2* mutants (figure 17). The excretory canal cell is the largest mononucleate cell in the animal with a width that varies between 1-2\(\mu\)m in wildtype cells (Lubarsky and Krasnow 2003) while in *fhod-2* mutants, they have been observed to be as wide as 4\(\mu\)m. The excretory canal cell is a dynamic cell which is constantly changing and adapting as the worm grows and moves. Problems in actin polymerization/nucleation could account for cells that terminate prematurely or for unregulated lumen size.
Figure 17: *fhod-2* mutants have defects in the excretory canal cell.

(A, D) shows a wildtype excretory canal cell with width of 1.34μm (width may vary but usually less than 2μm) along with corresponding cartoon depiction. (B, E) shows a mutant (M+Z-) worm with an abnormally wide lumen of 4.07μm. (C, F) shows mutant with an early termination/migration defect. Black triangle indicates location of gonad.
2.2.6 *fhod-2 (tm2133)* may have a role in the nervous system:

To test whether *fhod-2* has a role in neuronal development, we used various methods to visualize classes of neurons. DiI, is a vital fluorescent marker which stains the amphid neurons. These neurons help the worm sense its environment and move properly. The DiI staining revealed that the *fhod-2* mutation may cause defasciculation of neurons, improper migration and termination. I have used other neuronal markers to show that other neurons are affected. *odr-1, mec-4* and *sax-3* GFP reporters also revealed improper neuron formation and termination (figure 18). These head neuron defects may help to explain why mutants appear to have a foraging defect as these animals tend to dwell (figure 19). Normal *C.elegans* worms display two types of movements, foraging and dwelling. Foraging involves using its sensory neurons to explore its environment and search for food. Animals that dwell tend to stay in the same spot and do not exhibit the same exploratory behavior. To test if the lack of movement observed in the *fhod-2* mutants is due to dwelling or uncoordinated (unc) behavior where worms lack the ability to move, worms where placed on an empty plate (no food) and filmed for 2 hours. In wildtype worms, I observed that the worms will forage around the entire plate, in a constant state of motion exploring its environment. In the mutant worms, movement was limited and they remained relatively stationary compared to their wildtype counterparts. Even on food, mutants display limited movement. Mutant worms will move if poked or the plate is tapped indicating that they do not lack the ability to move. This suggests that they may display slightly unc or perhaps a lazy unc phenotype as they are capable of displaying the characteristic sinusoidal-like moving pattern of wildtype worms. When
mutants are put in liquid M9 buffer (thrashing assay), they do not trash as vigorously as their wildtype counterparts (figure 20). This is simply due to fatigue as when the worms are put back on a plate they have the ability to move right away. Another plausible reason for the lack of movement or low endurance observed in these animals is the fact that actin is basic building block for muscle fiber formation. If the mechanism for building actin fibers is impaired then it should be no surprise that movement is also impaired. Mammalian FHOD1 has been found to be highly expressed in aortic and coronary smooth muscle, and disruption of FHOD-1 activation has been found to influence smooth muscle cell tone and migration (Wang et al, 2004). It is possible that fhod-2 mutants have muscle defects that are contributing to this lazy unc phenotype.

Another method to visualize where the fhod-2 is expressed is with a via a fhod-2 promoter construct tagged to GFP fluorescence to see where the transcript (mRNA) is being expressed. Consistent with previous findings we see GFP expression in the excretory canal cell as well as neuronal expression. Preliminary results suggest that the sensory and touch neurons may also be impaired in fhod-2 mutants.
Figure 18: *mec-4::RFP* reporter constructs revealing touch neuron defects in *fhod-2* mutants.

Top shows wildtype *mec-4::RFP* showing 3 of the 6 touch neurons. *fhod-2* mutants reveal abnormal axon branching defects and blebbing. See inserts in image for close up of defect. White triangles indicate gonad.
Figure 19: *fhod-2* mutants appear to have neuronal defects and tend to dwell.

Top: DiI staining of wildtype and *fhod-2* mutant (*M+Z-*) neurons along with corresponding cartoon depiction. DiI stains for amphid neurons, which are used in the worms for chemosensory purposes. (A,B) shows wildtype pair of neurons with cell bodies while (C,D) shows *fhod-2* mutant with defasciculation of top neuron pair. Bottom: defects seen in neurons may attribute to the tendency of *fhod-2* mutants to dwell (both on and off food) compared to wildtype animals. Images taken from plates with a single worm roaming overnight, black line traces path of worm with green dot indicated where worm was placed and red dot indicating where worm was found the next day.
Figure 20: Graph comparing wildtype, *fhod-2* mutants and rescue line strains in thrashing assay.

Single worms were put into a drop of M9 minimal buffer and filmed for 5 minutes. Worms that were motile after the 5 minute periods were scored as thrashers. Error bars indicate the SEM and significant differences from the wildtype are indicated above bars as well as pairwise comparisons of various strains. IC826 is *mIs10/fhod-2 9.7kb rescue line* and IC861 is N2 over-expressing *fhod-2*. N = 100 for each strain, ***P<0.001.
2.2.7 *fhod-2* RNAi recapitulates the *fhod-2(tm2133)* phenotypes:

Further evidence to support that lack of *fhod-2* is the cause of the phenotypes reported above, we have been able to replicate our results using *fhod-2* RNAi. Previous RNAi work was done using a different fragment (pIC111 by Marie Evangelista and pIC 265 by Jadine Paw). I chose to focus on the FH2 region as this is the proposed region for formin function. By knocking out this region I hope to replicate the adverse effects observed in the *fhod-2 (tm2133)* mutants. RNAi experiments have proven to be consistent with these findings as we observe embryonic lethality, gonadal and excretory canal defects. Fat head or pinched nose defect is also observed in both the mutant and RNAi treated worms (figure 21), but the rate of occurrence is not as strong as defects seen in the gonad or excretory canal cell (figure 22). An example where the *fhod-2* RNAi was able to replicate the mutant phenotype is with embryonic lethality. In wildtype embryos, there should not be any embryonic lethality; however after *fhod-2* RNAi feeding the embryonic lethality rate increased to 16% (N=907). Embryos died in a similar manner as *fhod-2* mutants, displaying both gastrulation and cytokinesis defects. I also tested this on a more RNAi susceptible sensitive strain *rrf-3* (pk1426, II). This strain itself is somewhat sickly with a baseline embryonic lethality of 7% (N=254) but after treatment with RNAi lethality rate rose to 17% (N=229). Both wildtype worms and the *rrf-3* strain (strain that isi more sensitive to RNAi) displayed similar phenotypes after RNAi treatment.
Figure 21: *fhod-2* mutants appear to have a fat head or pinched nose defect.

Image on left shows a tapered head of a wildtype worm. The two images on the right show a *fhod-2* mutant (*M+Z-* ) and a wildtype worm after *fhod-2* RNAi show a fat head with a pinched nose phenotype.
Figure 22: Summary of defects on wildtype worms after fhod-2 RNAi.

Wildtype animals rarely have any defects and can be scored as <1% (N=100). After treatment of RNAi percentage of embryonic lethality is 16% (N=907). Percent of head defects 6%, gonad defects 18% and excretory canal cell defects 16%, N=136.
2.2.8 FHOD-2 is expressed in the pharynx and leading cells

To understand where FHOD-2 functions I used FHOD-2 antibodies and FHOD-2::GFP transgenic lines to look at FHOD-2 expression patterns. Our FHOD-2 antibody could not detect endogenous FHOD-2 \textit{in situ}, but was capable of detecting FHOD-2 in transgenic lines that over-expressed FHOD-2. It is possible that the endogenous levels of \textit{fhod}-2 are very low as it is not detected in situ antibody staining nor on western protein blots.

I showed that FHOD-2::GFP and over-expressing \textit{fhod}-2 genomic is expressed in the embryo and during early larval stages, in the leading cells and pharynx respectively, consistent with its role in embryogenesis, and especially during elongation (figure 23). However, I have not noticed any corresponding pharyngeal defects in \textit{fhod}-2 mutants.

Evidence that supports the role of \textit{fhod}-2 being involved early on during embryogenesis comes from western protein blotting. FHOD-2 protein was detected in the over-expressing lines from in mix stage animals (figure 24). Similar to the antibody staining, endogenous levels of FHOD-2 protein was not detected.
Figure 23: FHOD-2 expression and localization on over-expressing transgenics.

Top panel shows worm embryo on lying on its side, FHOD-2 expression (green) is detected in the pharynx region (A), anti-MH27 staining the adherens junctions in red (B) and a merged image is shown in (C). Bottom panel shows side view with punctate FHOD-2 expression in the pharyngeal primordium (E) (asterisk) of worm and ventral view of embryo shows FHOD-2 expression in leading cells (F) (white arrows) along with corresponding DIC images (G,H). Anti-FHOD-2 (green).
Figure 24: Western blotting with purified FHOD-2 antibodies is able to detect over-expressing FHOD-2 protein at the expected size of 93KDa.

(1) N2 mixed stage, (2) IC 825: N2 overexpressing line, *quEx220* embryo prep, (3) IC825: N2 over-expressing line, *quEx220* mixed stage, (4) IC 861: N2 over-expressing line, *quEx236* embryo prep, (5) IC861: N2 over-expressing line, *quEx236* mixed stage, (6) IC862: *mIs10/fhod-2* rescue line, *quEx237* mixed stage, (7) IC827: *mIs10/fhod-2* rescue line, *quEx221* mixed stage (8) IC828: *mIs10/fhod-2* rescue line, *quEx222* mixed stage. Expression of FHOD-2 was detected in the N2 over-expressing lines (lane 3,5) and faintly in the *mIs10/fhod-2* rescue line (lane 7) suggesting that endogenous levels of FHOD-2 protein are low. Probed with Anti-FHOD-2 (1:500) and secondary goat anti-rabbit HRP (1:5000). Expected size of FHOD-2 is 93KDa indicated with a bracket.
2.2.9 Injection of a fhod-2 genomic fragment has dominant effects and can partially rescue fhod-2 (tm2133) phenotypes

Ideally, to prove that it is indeed the fhod-2 deletion that is responsible for all of the defects observed, it would make sense that by replacing the missing gene via microinjection we would get reversal or “rescue” of the defects. Of the six rescue lines created, 3 showed significant FHOD-2 rescuing activity. I amplified 9.7kb full length fhod-2 from genomic DNA (oIC 257/260 stitch with oIC 259/427) to be microinjected into the gonad. I had partial success in being able to reduce the percentage of embryonic lethality (17.7% to 8.5%, P<0.05). I also see a decrease in sterility in the fhod-2 mutants, however fhod-2 (M-Z-) progeny were still 100% lethal (figure 25). I also noted a reduction in the observed mutant phenotypes; namely head, gonad and excretory cell defects (figure 26). However, we could not get full rescue of the phenotypes. It was suggested that perhaps the 9.7kb fragment was missing a part of the promoter region, so a new construct that contained a larger (5kb) piece of the promoter region was created and co-injected with the 9.7kb fragment. Again, results indicated that there was partial success in reducing embryonic lethality (17.7% to 11.5%, P<0.05) as well as a reduction in the previously mentioned phenotypes. However these differences were not significantly different between the 9.7kb injection piece and the larger piece. See table 1 and figure 27 for summary of results. It is difficult to determine the exact concentration of injection mixture required, as too much or too little can have the same adverse result. This may explain why there is not complete rescue. Over expression of the fhod-2 gene in a wildtype background can cause similar phenotypes as the seen in the mutants. We went
systematically from a standard concentration of 30ng/µL to further dilutions of 1:500 to 1:25000. Results suggest that a very small quantity of *fhod-2* is required for proper development.
Figure 25: Percentage of embryonic lethality between various rescue lines.

The baseline lethality rate for mutants is 17.7%, we were able to significantly lower the lethality rates to 8.5%, 11.5% and 9.3% in the rescue lines. Differences in percentage between the various rescue lines are not significant. Error bars indicate the SEM and significant differences from the fhod-2 mutant are indicated above bars *P<0.05. Table compares various rescue lines and percent lethality.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Percent Embryonic Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC710: mIs10/fhod-2 (N = 1368)</td>
<td>17.7% Lethality (28.6 STD, 3.8 SEM) Used as baseline for comparison for IC710 rescue lines.</td>
</tr>
<tr>
<td>IC826: mIs10/fhod-2, quEx219 (N = 616)</td>
<td>8.5% Lethality* (11.2 STD, 1.8 SEM)</td>
</tr>
<tr>
<td>IC857: mIs10/fhod-2, quEx232 (N=800)</td>
<td>11.5% Lethality* (19.4 STD, 3.7 SEM)</td>
</tr>
<tr>
<td>IC827: mIs10/fhod-2, quEx221 (N=172)</td>
<td>8.7% Lethality (10.3 STD, 3.6 SEM)</td>
</tr>
<tr>
<td>IC862: mIs10/fhod-2, quEx237 (N=103)</td>
<td>10.7% Lethality (12.1 STD, 5.4 SEM)</td>
</tr>
<tr>
<td>IC863: mIs10/fhod-2, quEx238 (N=791)</td>
<td>14.2% Lethality (17.4 STD, 3.2 SEM)</td>
</tr>
<tr>
<td>IC828: mIs10/fhod-2, quEx222 (N=163)</td>
<td>9.3% Lethality* (16.5 STD, 4.1 SEM)</td>
</tr>
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Table 1: Percentage of embryonic lethality between various rescue lines.

Table compares various rescue lines and percent lethality. Corresponds to figure 25 graph. *P<0.05.
Figure 26: Comparison of head, gonad and excretory canal cell defects observed in *fhod-2* mutants and over-expressing lines.

Gonad defects are the most prevalent, followed by the excretory canal cell and head defects. Head defects were observed at 12%, 10%, 6% and 12%, gonad defects at 70%, 27%, 54% and 57% and excretory canal cell defects at 44%, 20%, 32% and 21% in the *fhod-2* mutants (IC710) (N=50), the N2 over-expressing *fhod-2* line (IC861, quEx236) (N=30), the *mIs10/fhod-2* rescue line (IC26, quEx219) (N=50) and the *mIs10/fhod-2* 2 piece rescue line (IC857, quEx232) (N=42) respectively. A reduction in defects was seen in both rescue lines. Note that wildtype animals are not shown. Head, gonad and excretory canal cell defects are very rare in wildtype animals. In a wildtype population we expect >1% defects.
Figure 27: Sterility in *fhod-2* mutants versus mutants from rescue line.
66% of *fhod-2* mutants are sterile, this number decreases to 35% after rescue microinjection with a 9.7kb *fhod-2* fragment ((IC826, quEx219). Error bars indicate the SEM and significant differences from the *fhod-2* mutant are indicated above bars, N = 115 for each strain, ***P<0.001.
Chapter 3
Discussion

Previous studies on formins provide a broad overview of the types of defects that can arise from unregulated or missing formin function. It is clear that formin function is incredibly diverse and may play unexpected roles in the cell. My thesis was to characterize the \textit{fhod-2} gene in \textit{C.elegans}. At the moment the formin \textit{fhod-2} cannot be categorized into a single role. It appears that the phenotypes of the \textit{fhod-2} mutant are pleiotropic. Embryos suffer from gastrulation, elongation and cytokinesis defects and any mutants that manage to survive into adulthood suffer from a whole host of defects. The fact that we see escapers in \textit{fhod-2} (M+Z-) animals is probably due to the fact that there is a maternal contribution of gene product or protein. \textit{fhod-2} (M-Z-) animals are lethal as there is no gene product from the mother to compensate for the loss. It is plausible that a common bond between all the observed defects is the requirement of actin and formin actin regulation for proper formation and development.

3.1 \textit{fhod-2} is necessary early on in the cell for proper development

\textit{C. elegans} is predicted to have 6 formins, of these only one has been studied, \textit{cyk-1} (CYtoKinesis defect-1). \textit{cyk-1} is part of the Dia family of formins and is a recessive maternal effect lethal gene. Genetic analyses from fungal species to yeast have revealed the link between the Dia formins and cytokinesis as they are responsible for cellularization (Severson \textit{et al}, 2002, Swan \textit{et al}, 1998). \textit{cyk-1} (M-Z-) mutants cannot produce oocytes due to defects in cellularization, in weaker mutant alleles embryos are produced but they fail to fully divide resulting in a multinuclei phenotype (Liu \textit{et al},
CYK-1 protein has been shown to co-localize with actin microfilaments at the leading edge of the cleavage furrow (Swan et al, 1998). Loss of function studies in Drosophila also reveals a failure in cell division and multinucleated cells (Castrillon and Wasserman 1994, Goode and Eck, 2007). Mutant Dia animals are sterile and some are lethal. Further evidence comes from analysis of cytokinetic actin ring via electron microscopy which revealed barbed end filament assembly and linear unbranched bundles of actin filaments (Castrillon and Wasserman 1994, Noguchi and Mabuchi 2001). This is consistent with the method of formin filament assembly. Ablation of Arp2/3 activity in both C.elegans and Drosophila did not impair the formation of the cytokinetic ring or cause cytokinesis, further supporting the role of formins (Evangelista et al, 2002).

Analyses of other model organisms have revealed that formin mutations also lead to cytokinesis defects which suggest a conserved and essential role for formins in regulation of the actomyosin contractile ring. fhod-2 (M-Z-) mutants also share a similar defects as cyk-1(M-Z-) mutants. There is a variable failure of oocyte maturation meaning that mutants are usually sterile but when embryos do develop they have a multinuclei phenotype. It is possible that this is the result of a defective plasma membrane enclosure of the ooctyes or assembly of the contractile ring. The actin filaments forming this cleavage plane could be shortened or unable to form the proper adhesion causing the failure to total cellular ingression. The defects seen in fhod-2 embryos could be due to a variety of defects all owing to the failure of properly regulated actin. fhod-2 could function similar to cky-1, where its role is in the cytokinetic actin ring. Or perhaps the failure of the cell to properly divide could be due to inappropriate division of cellular
content. Cell polarity regulates the integrity and future orientation of cells and specific cellular cues are needed to form the polar ends of the embryo. In order for cells to ingress, actin myosin has to become enriched at the apical surfaces which require polarities that distinguish their different surfaces (Rohrschneider and Nance 2009). It is plausible that if there was a failure in the polarization of the cell, actin would not be properly localized and cellular division would be defective.

\textit{fhod-2} (M+Z-) mutants do not display early cytokinesis defects, however some embryos die during gastrulation or elongation. This suggests a maternal effect, allowing the embryos to undergo proper cytokinesis. However, it is possible that quantities provided are not sufficient to support the full development of the embryo and results in embryonic death. The role of actin has been shown to be indispensible for dorsal and ventral enclosure of the hypodermis and elongation in the embryo (Faix and Grosse 2006, Wood and Martin 2002). Dia has been shown to localize in the leading edge adherens junctions during dorsal enclosure in \textit{Drosophlia} (Homem and Peifer 2008). This is consistent with the defects seen in \textit{fhod-2} as mutants having trouble going through gastrulation or failing to elongate. Antibody staining detects FHOD-2 support these findings as FHOD-2 is detected in the leading cells. Leading cells migrate towards each other enclosing the embryo, these cells are guided by filopodia. Filopodia are cell surface protrusions composed of bundles of parallel actin bundles that the cell can use to sense its environment (Wood and Martin 2002). Again, loss of function studies have eliminated the role of the Arp2/3 complex and its activators for assembly of filopodia actin filaments.
(Steffen et al, 2006). Instead, emerging evidence implicates formins for this role. It is plausible that the \textit{fhod}-2 mutants have defective or perhaps lack sufficient filopodia structures to act as cues to guide cell migration or cell adhesion. Or perhaps the mutation causes excessive actin filaments to be formed as there is no regulation, leaving the cells “locked” in place and unable to undergo the necessary modifications. Homem and Peifer (2008) found that reducing levels of Dia in \textit{Drosophila} lead to extended abnormal cell processes. During enclosure of the embryo, filopodia are usually sent out to guide cell fusion but in mutants they were longer and more haphazardly placed causing disruption of the tissue interface. Another formin called INF2 has been found to both promote actin filament assembly and demolition. It is thought that this switch between the on and off state is dependent on age of the filament (Chhabra and Higgs 2006). Put into the context of cellular remodeling, switching off formin activity may allow cells to move and reposition themselves.

\subsection*{3.2 \textit{fhod}-2 may be necessary for organogenesis}

Across species, the importance of formins has been solidified in the proper formation and regulation of tubular shapes. Formins have been widely found to play a role in organogenesis from mouse to humans and is expressed in various tissues such as the heart, kidney and brain. It is possible that a potential role for \textit{fhod}-2 is in development and maintenance of tubular organs. Most major organs such as the lungs, kidney and vasculature are composed of tubes; they are a fundamental unit of organ design. They act to transport gases and liquids from one cell to another throughout the body. These tubes are composed of cells connected together to form a sheet of epithelium and wrapped into
a tube. How cells are assembled into tubes and how tubule shape and size are regulated is still relatively unknown. An understanding of these processes would be vital in medicine as many human diseases are essentially due to failure in regulation such as polycystic kidney disease and atherosclerotic heart disease. The formation and regulation of tubal size is crucial for proper development and function in tissues and organs.

*fhod-2* may have a role later in development for gonad and excretory canal cell formation. The somatic gonad sheath as well as the excretory canal cell is not properly formed, both are examples of tubal formation gone wrong. DIC imaging has revealed improper migration and branching defects in both of these structures. In the excretory canal cell we have also seen enlargement in the lumen. The excretory canal cell is connected to the hypodermis via gap junctions as well as through interactions with the hypodermal basement membrane, its growth occurs evenly to match the elongating animal (Buechner 2002). The length of the canal can be affected by early development as the initial growth of the canal cells begins during embryogenesis. It is thought that proteins link the actin cytoskeleton to the apical cell membranes. The specific functions and interactions of these proteins in tube morphogenesis are not yet known, however results suggest that proteins at the cytoskeleton and luminal surface stabilize lumen structure and limit its growth (Lubarsky and Krasnow 2003). Lumen may be formed by the coalescence and fusion of the apical membrane vesicles. Lumen forms within the cytoplasm due to cell hollowing (Buechner 2002, Lubarsky and Krasnow 2003). A dozen genes, such as members of the *exc* (Excretory Canal Abnormal) family of genes
have been found to affect the size and shape of the excretory canal cell, which resulted in enlarged tubules (Buechner et al, 1999). For example, exc-4 has been found to be required for the precise control of diameter and loss of function may cause the lumen to collapse or swell (Berry et al, 2003). exc-4 is the C.elegans ortholog to the CLIC (Chloride intracellular channels) proteins in humans (Berry et al, 2003). CLIC proteins are found in most intracellular membranes and are integral components of the chloride channel. They are thought to operate as ion shunts to equalize electrochemical potentials and many are associated with cytoskeleton proteins, but their cellular roles and associations of these proteins are not known (Berry et al, 2003, Paul and Beitel 2003). It is hypothesized that these channels are regulated by actin as CLIC5 and CLIC1 channel conductance can be reversibly inhibited by F-actin and can reversed by using cytochalasin to disrupt the F-actin (Singh et al, 2007) Actin-regulated membrane CLICs could modify solute transport at key stages during cellular events such as apoptosis, cell and organelle division and fusion, cell-volume regulation, and cell movement (Singh et al, 2007). As formins regulate both actin filament construction and deconstruction, it is possible that they may have a role regulating these channels. Unregulated electrochemical potentials could be creating a salt imbalance, causing the lumen to swell/enlarge as observed in fhod-2 mutants.

Other studies have implicated formins in tubule regulation. Formin IV, a member of the formin gene family encodes mouse limb deformity gene products. Disruption of this gene results in agenesis of the kidneys, early failure of ureteric bud outgrowth as well
as branching defects (O’Rourke et al, 2000, Wynshaw-Boris et al 1997). These authors suggest that formin proteins are required for early developmental tubule formation in the kidney. In *C.elegans* the excretory canal cell is analogous to the kidney and as previously mentioned, *fhod-2* mutants are observed to have morphology defects in this cell. As the worm reaches senescence, it is known that the excretory canal cell tends to lose elasticity and will shorten (Buechner 2002). However, in mutants defects are observed in early adulthood. It is possible that the *fhod-2* mutation may be hastening the natural deterioration of the organ. The enlarged lumen and “wavy” appearance of the cell may be due to the loss of elasticity in the organ. Two other formins, *form-3* and *DAAM* have been implicated in tracheal development in *Drosophila*. Tracheal cells invaginate from the epidermis, migrate, change shape and branch to form a tracheal network. Some of the branches fuse with adjacent branches to form a continuous luminal network. Anastomosis is mediated by a cytoskeleton track containing F-actin, microtubules and E-cadherin complexes which forms between fusion cells (Lee *et al*, 2003, Tanaka *et al*, 2004). Fusion cells extend filopodia to contact partners in adjacent branches and mediates lumen formation. Using GFP live imaging, it was shown that F-actin spans the adjoining fusion cells and mediates the luminal connection, when a mutation removes most of the FH2 domain abnormal anastomosis results (Tanaka *et al*, 2004). This study demonstrated the importance of a functional FH2 domain and that the *form-3* formin has a role in assembly of the actin-rich track that is essential for cellular rearrangement during tracheal fusion. I have shown through using *fhod-2* RNAi which knocks down most of the FH2 domain, that this region may be important for proper gonad development. After being
subjected to \textit{fhod}-2 RNAi, wildtype worms show similar branching and migration defects as mutants. It is possible that the branching defects in \textit{fhod}-2 mutants are a result of adjoining cells failing to fuse together due to actin defects. Other studies have implicated \textit{DAAM} as being necessary for keeping the tracheal tubes from collapsing. \textit{DAAM} has been shown to co-localize with apical actin, mutations lead to discontinuities in the tubular network and an undefined taenidial fold pattern of the tracheal cuticle, which results in tracheal tube collapse (Masurek et al, 2005). \textit{DAAM} mutants have reduced apical actin levels, completely abolished actin organization and feature short, thin cross linked cables compared to wildtype (Masurek et al, 2005). These authors have suggested that the major role of \textit{DAAM} is to organize the actin filaments in lieu of simply actin nucleation or it is entirely possible that \textit{DAAM} is primarily required for actin polymerization but tightly coupled to an actin "organizing" protein. Both genes reveal a key a role of formins and the FH2 domain for regulating the size and shape of the tubules. In \textit{fhod}-2 mutants, both the tubule gonad and excretory canal cell are observed with morphology defects. Given the established role of formins, it is possible that \textit{fhod}-2 plays a role in regulating the shape and size of the somatic gonad sheath and excretory canal cell. The \textit{fhod}-2 promoter driven GFP construct is consistent with this observation, as GFP is expressed in the excretory canal cell. We do not observe any expression in the gonad and this may be due to the fact that \textit{fhod}-2 does not act directly on the gonads but acts on surrounding tissues or cells that in turn influence gonad development.
Cell polarity is also a requisite for proper tissue formation, and is especially important during organogenesis as oriented divisions contribute to organ size and shape (Baena-López et al 2005, Strutt 2005). An improper division of cellular content during the early cell divisions could manifest itself later on during development. For example, early cell divisions in the developing *Drosophila* wing are highly oriented and correlate with the growth of the long axis of the organ (Baena-López et al 2005). Mutations in the cell polarity genes *dachsous* (ds) and *fat* (ft) abolishes ordered orientation of cell division and results in shorter and broader wings in *Drosophila* (Strutt 2005). The defects seen in the gonad and excretory canal cell vary in severity and are not 100% penetrant, therefore they may originate early in the development of the embryo and predispose the worm to the observed future defects. The defect seen in abnormal gonad migration may be due to a lack of polarity cues.

### 3.3 fhod-2 mutants are sterile

*fhod-2* (*M+Z-*) mutants that survive into adulthood are usually sterile, as they do not go on to produce a viable progeny. Several genes associated with actin have been correlated with sterility. In *C.elegans*, maturing oocytes are connected to the younger population of germ cells at various stages of development via cytoplasmic bridges (Wolke *et al*, 2007). The *C.elegans* gene *cyk-1* is required to form and organize these membrane invaginations to separate germline nuclei and hermaphrodites possessing strong *cyk-1* mutations cannot produce oocytes as a result of the defective cellularization (Swan *et al*, 1998). As *fhod-2* shares an overall 27.5% sequence similarity to *cyk-1* (BLASTP 2.2.20+) it is imaginable that they may have some overlapping functions.
Similarly, in *Drosophila*, actin microfilaments mediates partitioning of cytoplasm and genetic material in both oogenesis and spermatogenesis, it has been shown to be essential for fertility (Castrillon and Wasserman 1994). The developing egg chamber has ring canals (which contain specialized F-actin) that are necessary to mediate the dumping of nurse cell contents into the oocytes (Sutherland and Waite 1999). Sterility observed in the *fhod-2* mutants could be due to the fact that there is improper cellularization or partition of nutrients being incorporated into the developing *C.elegans* germ cells. In contrast to *Drosophila*, *C. elegans* has no known distinct nurse cells as all germ cells have the ability to be fertilized (Gumienny *et al*, 1999). The germ cells may contribute materials to the oocytes, acting transiently as nurse cells before becoming gametes themselves (Cooley and Theurkauf 1994). Therefore it is thought that developing oocytes get the proper nutrients from actin mediated cytoplasmic streaming which allows incorporation of core cytoplasm synthesized earlier on in development (Wolke *et al*, 2007). Streaming serves to distribute various morphogenetic determinants and intracellular components such as yoke proteins and mRNA (Liu *et al*, 2007). Oocytes are associated with a mesh of actin and streaming is thought to be driven by actin and myosin-dependent forces generated in the enlarging oocytes themselves or adjacent oocytes (Wolke *et al*, 2007). The exact mechanism is not known at this time, but actin plays a large role as these authors observed a lack or aberrant streaming when gonads were treated with actomyosin cytoskeleton inhibitors. A defect in cytoplasmic streaming could explain the sterility observed in the *fhod-2* mutants. I have attempted to determine if this was the cause using the same protocol described by Wolke *et al*, 2007.
Unfortunately, it was not entirely successful, future work could be done to refine this technique to determine if \textit{fhod-2} mutants have a defect in cytoplasmic streaming.

Actin has also been implicated in many nuclear functions such as transcription, mRNA processing, chromatin remodeling and nuclear matrix association (Bettinger et al, 2004, Farrants 2008). Many members of the ARP (actin related proteins) family of proteins are localized in the nucleus. ARPs have been found to be a subunit of chromatin-modifying complexes, involved in chromatin modification, DNA repair and transcription regulation and are required by all three RNA polymerases (Chen and Shen 2007, Olave \textit{et al}, 2002). Although the mechanism behind actin involvement in these functions is still not clear. Arp4 in particular has the ability to interact, bind and modify histones. It maintains integrity and regulates the dynamics of the chromatin modifying complexes and targets them to chromatin via its interaction with core histones (Chen and Shen 2007). Arp4 and actin are found together suggesting they physically form a dimer. Recall that Arp2 and Arp3 combine to form one of the most common actin nucleators in the cell, so it is highly possible that other members of this family functions in a similar manner. \textit{fhod-2} may act similarly to Arp4 as DAPI and histone (\textit{ruIs32}) markers reveal that organization at the most basic DNA level is impaired. While the DNA is present, it is not organized properly onto histones, which requires actin to properly coil. This could account for sterility seen in the \textit{fhod-2} mutants.
3.4 fhod-2 may have a role in neuronal formation

As neural guidance and outgrowth utilizes many of the same cues and mechanisms used in excretory canal cell extension it may not be a surprise that fhod-2 mutants have neuronal defects (Beuchner 2002). To determine if fhod-2 was also necessary for proper neuron formation, odr-1, mec-4 and sax-3 GFP reporter genes were injected into the mutant strain. Improper neuron formation, termination and branching were observed. odr-1 (ODoRant response abnormal) is expressed in chemosensory neurons with the strongest expression in the AWC and is required for normal responses of environmental odorants. mec-4 (MEChainosensory abnormality) encodes an amiloride-sensitive Na+ channel protein (degenerin) required to sense gentle mechanical stimuli (such as touch) along the body wall, it is expressed in all 6 touch neurons. sax-3 (Sensory Axon guidance) is an axon guidance receptor belonging to the ROBO (Roundabout) family and is expressed in many neurons and some hypodermal and muscle cells. Preliminary results reveal that neuron formation and migration are impaired in mutants. Early termination, branching and “blobby neurons” are observed in the mutants. Deficits seen in these neurons could correlate to defects in movements revealed in both movement and thrashing assays. Normally, wildtype worms display roaming and foraging behavior on the plate, which is partially accomplished via sensory neurons providing cues about the environment. In the fhod-2 mutant worms, they tend to dwell which could be due to the lack of sensory feedback from the environment. These worms are fully capable of moving when the worm is gently poked or the plate is tapped therefore, dwelling may be due to a slight lazy uncoordinated (unc) defect. Mutants also
have low endurance compared to wildtype as shown by thrashing assays. Actin rich filopodia have been implicated as sensory tools to explore environmental cues to guide cell axon extension (Dent and Gertler 2003, Wood and Martin 2002). As formins have been implicated to be important in nucleating filopodia formation, it is possible that fhod-2 mutants lack the filopodia to receive feedback from the environment, giving rise to defective neuron formation. In turn, the worms themselves lack the proper chemosensory neurons for proper behavior leading to the unc behavior.

FHOD-2 is a homolog of the DAAM subfamily. The DAAM subfamily have been associated with proper neural growth, as they are reported to be abundant in the developing nervous system. Drosophila formin dDAAM has been found to play a critical role in axonal morphogenesis, it is required for filopodia formation at the growth cones (Matusek et al, 2008). Growth cones regulate axonal growth control and help axons reach their target site. Immunostaining experiments show that dDAAM strongly co-localizes with actin and loss of function experiments show both a reduction in number and length of filopodia (Matusek et al, 2008). This suggests that dDAAM is required for elongation which is a typical formin role. With less or shorten filopodia it is not surprising to see fhod-2 mutants with axon defects such as early termination or over-extension. It is also interesting to note that DAAM orthologs has been shown to be expressed at elevated levels in the nervous system of many other species such as chick, mouse and Xenopus, it may represent an evolutionary conserved function of regulated axon growth (Matusek et al, 2008, Nakaya et al, 2004). In fact, (Matusek et al, 2008) showed that Drosophila
*dDAAM* mutants can be rescued using mouse *DAAM*. Further evidence for the role of actin nucleators in neural formation comes from cordon-bleu which functions similarly to formins by forming linear actin filaments. Cordon-blue has been shown to be important for neuronal morphology and development. Studies show a dose dependant increase in rate of assembly of filaments and increased expression enhances neural formation while depletion inhibits dendrite branching (Ahuja et al, 2007). Neuronal defects in *fhod-2* mutants are not inclusive and seem to affect certain neurons suggesting that formins or actin nucleators may only play a partial role as there are many other genes that have been shown to be essential for axon growth. Members of the *Rac* family have been shown to be essential for axon growth so it may overlap with formin function as reduction of *Rac* function correlates with enhanced axon growth defects of *DAAM* mutants and vice versa (Hakeda-Suzuki et al, 2002).

### 3.5 Rescue experiments partially restores *fhod-2* function

Rescue experiments have restored some local order in these structures but overall fail to mimic the highly ordered structures seen in the wildtype worms. Complete rescue may be difficult as a precise level of *fhod-2* is crucial for worm development. However, combined results from expression patterns, RNAi, a *fhod-2::gfp* transcriptional reporter and rescue experiments suggest that the observed phenotypes are indeed due to the *fhod-2* (*tm2133*) deletion. Our findings suggest that *fhod-2* is required in multiple stages of development, early on for proper embryonic cell divisions and morphogenesis as well as later on for development of the gonad, excretory canal cell and neurons. To provide further evidence that the phenotypes associated with the *fhod-2(tm2133)* deletion is
indeed due to the *fhod*-2 deletion (as opposed to another mutation) we planned to rescue these phenotypes with a wildtype *fhod*-2 copy of the gene. I have amplified the full length *fhod*-2 gene and injected this into the mutant line. While I have not been able to achieve full rescue of the mutant, I have had success in significantly reducing many of the observed phenotypes. A decrease in embryonic lethality, sterility as well as morphology defects in the head and gonad suggest some degree of rescue. Since I could not completely rescue the phenotypes, it was thought that either the concentrations of *fhod*-2 were too high/low or perhaps part of the promoter region of *fhod*-2 was missing when the construct was created. In order to rule out these possibilities, different rescue lines with varying injection concentrations were created using two different sizes of the *fhod*-2 promoter region. These lines have not yielded any significant difference in rescuing the phenotypes. While many studies have used the injection method for rescue of mutants successfully, this method does not always result in complete rescue (Matusek *et al* 2006). Complete rescue may not be possible due to a variety of reason. It may be due to the fact that I have not been able to determine the exact amount of gene product needed in the cell as previous studies have indicated that the lack or over-expression can both lead to adverse effects and even lethality (Habas *et al* 2001, Matusek *et al* 2006). A very fine balance is needed, otherwise too little or too much can result in similar phenotypes as a null mutant. It is also possible that we have not been able to achieve full rescue may also be due to the fact that injecting *fhod*-2 into the germline may be silenced. The germline is strictly controlled and perhaps the injected DNA is being recognized as foreign and is not able to be fully expressed.
While many of the phenotypes mentioned above may initially appear isolated from one another, there is an underlying link of improper communication and lack of regulation between the cells. Neurons stopping prematurely, gonads migrating in the wrong direction or cells failing to adhere properly indicate a failure of control or regulation. In all of these cases, the indispensible importance of proper actin recruitment and organization can be attributed to the defects seen. Many studies have implicated the fundamental importance of actin for correct development. Actin is the central organizer of the cell. Its role in the cell is indispensible, ensuring proper movement, cell signaling and providing stabilization. Adverse effects can be caused by the lack or over-production of actin filaments. Accordingly, deficits in the machinery that is responsible for actin nucleation or organization will be detrimental to the cell. Various roles of formins and their influence over the actin cytoskeleton have been well established and the mutant fhod-2 phenotypes observed can be contributed to lack of formin function.

### 3.6 Future directions

Future work on fhod-2 will be focused at attempting full rescue of the observed phenotypes, this will involve using the germline specific pie-1 promoter to express fhod-2. This should result in better gene expression in the germline as pie-1 has been shown in previous experiments to be efficient in facilitating expression in the adult germline and promoting germ cell fate (Ghosh and Seydoux 2008, Tenenhaus et al, 2001). Our previous method of injection may not have been able to fully rescue due to the fact that injection of fhod-2 into the germline is weak or silenced. To test this theory I am in the
process of making a *pie-1* construct. The *pie-1* promoter has been shown to be able to bypass the strict regulation of the germline. By making a construct that contains this promoter with my gene of interest, I may be able to achieve improved *fhod-2* expression and rescue of mutants.

As the *fhod-2::gfp* promoter and other various neuronal markers have revealed defects in neuron migration and structure, the role of *fhod-2* in neurons is also an area that requires further exploration. The consequences of these defects are not entirely apparent at this moment and more testing will need to be done to see if they are detrimental to the worm. This will also shed some insight as to the source of unc behavior seen in the mutant worms and determine if it is indeed a neuron sensory defect or perhaps a defect in muscle contraction.

Yeast-2-hybrid experiments with *fhod-2* to determine interacting proteins or genes would be beneficial as elucidating a regulatory pathway for *fhod-2* would help to determine how it is regulated. It is already known from other studies with actin nucleators that Rho, Ena/Vasp, Wnt/Fz are potential interacting partners and should be tested first. Also it has been noted from our lab that *fhod-2* and *unc-34/Ena* mutants share similar phenotypes and may interact or perhaps share overlapping functions. The cell has many different formins and it has been suggested that formins are specialized, however studies have also shown that in many cases there exists cross talk between formins and a mutation in one formin can be compensated by another formin or actin nucleator. Future work creating different formin mutants using suppressors and enhancers should provide valuable information as to the degree of specialization of *fhod-2*. 
Lastly, unraveling the role of fhod-2 or formins in general will have a lot of impact on our understanding of analogous counterparts in the human system. It has been suggested that various actin nucleators may play a role in cancer and tumor progression. Three members of the diaphanous-related family of formins, DRF1-DRF3 have been found to be expressed in the highly invasive MDA-MB-231 breast adenocarcinoma cells (Lizárraga et al, 2009). This study implicates DRF proteins as essential components in the regulation of actin assembly during invadopodia formation. Invadopodia are actin-driven filopodia-like protrusions with matrix degrading activity that promote metastasis of tumor cells. Silencing or the absence of DRFs leads to a reduction in invadopodia, decreased matrix degradation and invasion capacity. In fact, analysis of gene expression revealed a significant 2-fold to 2.5-fold over-expression of DRF2 and DRF3 transcripts in breast tumors compared with normal human breast tissues. Formins may not be directly responsible for the formation of tumors but may help promote metastasis. The rate of formin activity in these cells may be what determines if a tumor remains benign or malignant. Regulators of actin nucleators have also been implicated in human cancers. For example, the Wnt signalling pathway is implicated in several types of human cancers such as colorectal cancer, head and neck carcinoma, melanoma and leukemia (Mazieres et al, 2005). Aberrant activation of this pathway leads to carcinogenesis. Recall that the Wnt signalling pathway was responsible for cell polarity, cell adhesion and motility. It is plausible that over-expression of Wnt also promotes metastasis of tumours. Fully understanding these pathways can lead to novel and specific targeted therapies in
treatment of diseases. Deciphering the role of \textit{fhod}-2 remains complex, but the enigma is beginning to unravel.
References


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