Essential functions of IFA-2 domains in Caenorhabditis elegans fibrous organelles

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entitled

Essential Functions of IFA-2 Domains in *Caenorhabditis elegans* Fibrous Organelles

by

Kyle Christopher Williams

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Biology

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May 2012
An Abstract of

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The *C. elegans* fibrous organelle (FO) consists of apical and basal
hemidesmosomes linked by cytoplasmic intermediate filaments (IFs). FOs are most
prominent in the epidermis where they overlie body wall muscle and serve to transmit
force from the muscle to the cuticle. IFA-2, one of four epidermally expressed IFs whose
loss results in epidermal fragility and failure of muscle-cuticle force transmission,
localizes to FOs. All IFs contain three domains: a central rod domain and globular head
and tail domains. The rod is essential for assembly of IFs into mature filaments while
less is known about the functions of the head and tail domains. Roles of the head and tail
domains of IFA-2 were examined by expressing GFP-tagged IFA-2 variants in transgenic
animals and examining their phenotype and localization in wild-type and null
backgrounds. An IFA-2 variant containing only the head domain (IFA-2H) was able to
localize to FOs on its own. High levels of larval-stage IFA-2H incorporation into FOs
correlate with a dominant-negative muscle detachment phenotype, while low-level
incorporation results in healthy animals. This suggests the head domain interacts with
FO components essential to tissue integrity. To determine possible interactions between
the head domain and FO proteins, the yeast two-hybrid system was used. An initial screen detected that the head and tail domains of IFA-2 are able to interact with each other. To obtain a more complete list of IFA-2H interactors, a *C. elegans* cDNA library was screened against IFA-2H. After PCR and sequencing of the 239 clones generated from the library screen, 45 unique proteins were found to interact with IFA-2H. Several criteria including functional roles, tissue expression data, and frequency of library inserts has been used to analyze the data and assign relevance to the interactions. One of the proteins found in the library screen, K04G7.1, was investigated further and found to localize throughout the pharynx where it may interact with IFs. This work should help to elucidate the role of the different domains of IFA-2 in FO function and reveal the underlying defects leading to tissue fragility.
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List of Abbreviations

β-gal .................... beta-galactosidase

cfu .................... colony forming unit
CTAB ............... cetrimonium bromide
DMSO .................. dimethyl sulfoxide
EtOH .................... ethanol
FO .................... fibrous organelle

gal .................... galactose
GFP .................... green fluorescent protein
GTE ................... glucose-tris-ethylenediaminetetraacetic buffer

IF ................... intermediate filament
IFA-2ΔH ............ IFA-2 lacking the head domain
IFA-2ΔT ............. IFA-2 lacking the tail domain
IFA-2H ............... the head domain only of IFA-2
IFA-2R .............. the rod domain only of IFA-2
IFA-2T ............... the tail domain only of IFA-2

LiAc .................. lithium acetate

NGM ................. nematode growth media

ONPG ............... ortho-Nitrophenyl-β-galactoside

PCR ................... polymerase chain reaction

raf .................... raffinose
RNAi ................ RNA interference

SDS ................... sodium dodecyl sulfate
SOC .................... Super Optimal broth with Catabolic repressor

TE .................. tris-ethylenediaminetetraacetic buffer
Y2H .................. yeast two-hybrid
YPD .................. yeast peptone dextrose growth media

-H .................. minus histidine media
-L .................. minus leucine media
-T .................. minus tryptophan media
-U .................. minus uracil media
1. Introduction

The nematode *Caenorhabditis elegans* is an excellent organism for studying the process of cell adhesion. *C. elegans* offers many advantages including a transparent body that allows for observation of cell position and the location of fluorescently-labeled proteins associated with adhesion structures, an invariant cell lineage making it easy to pinpoint defects in development; genetic advantages including a short life cycle, well-developed genetic techniques such as RNA interference (RNAi), cross fertilization, and generation of transgenes by microinjection of DNA (Sulston et al., 1983). Transgenes introduced by microinjection are typically incorporated into extrachromosomal arrays, which although subject to somatic loss (mosaicism) and germ-line silencing, give reliable expression of fluorescently-tagged proteins in the somatic cells of the worm (Hillier et al., 2005).

The short life cycle of *C. elegans* is about three days at 22°C (Byerly et al., 1976). After hatching, worms go through four larval stages before arriving at adulthood. Molting takes place at the end of each larval stage when new cuticle is synthesized by the hypodermis and the old cuticle is shed. The molting process consists of a lethargus stage, followed by the ecdysis or shedding stage. The hypodermis makes attachments to the cuticle through apical fibrous organelle receptors, and these cell-matrix attachments must
be remodeled at each molt. Embryogenesis consists of proliferation and organogenesis stages. Proliferation occurs during the first 330 minutes of development and includes the cell divisions from the single cell to 550 naïve cells. Organogenesis is the remaining embryonic development where the three germ layers differentiate and the embryo elongates to a three-fold stage before hatching. The three germ layers include the ectoderm (hypodermis and neurons), mesoderm (pharynx and muscle), and the endoderm (intestine). The hypodermis makes attachments to the cuticle through fibrous organelle adhesion complexes, and these cell-matrix attachments must be remodeled at each molt. Mature hermaphrodites develop 1090 somatic cells, 131 of which undergo apoptosis, to give 959 total cells. 302 of these are neurons along with 95 body wall muscle cells. The hypodermis consists of the main body syncytium (hyp7), 5 hypodermal cells in the head, and 4 more in the tail (Sulston and Horvitz, 1977).

There are two sexes of the nematode; hermaphrodites and males. Hermaphrodites allow for the potential to generate genetically identical progeny by self-fertilization. Mating through males allows standard genetic crosses to be carried out. Despite having more somatic cells, males are smaller than hermaphrodites. Males also possess a copulatory apparatus including the fan-like tail and spicules. Hermaphrodites contain a XX sex chromosomal pair, while males contain a single X chromosome. The lack of a second X chromosome in males is the result of spontaneous non-disjunction of the X chromosome during meiosis, and occurs rarely in hermaphrodite progeny at 0.1-0.2% of animals (Hodgkin and Doniach, 1997)

The body of the nematode has two compartments separated by a pseudocoelomic cavity. The outer compartment consists of the cuticle, hypodermis, excretory system,
neurons, and muscle, while the inner compartment contains the pharynx, intestine, and gonad. Neurons and hypodermis are separated from muscle by the basal lamina, with muscles receiving input from motor neurons. A cross-section of the worm may be seen in Figure 1.

**Fig. 1 – Cross section of C. elegans.** The inner compartment contains the intestine, ovary, and uterus and is separated from the outer compartment by the pseudocoelomic cavity. The outer compartment contains muscle and the epidermis (hypodermis) that are separated by the basal lamina. The fibrous organelle adhesion complexes are oriented circumferentially in hypodermis overlying muscle and transfer force to the cuticle. Reprinted with permission from Dr. John Plenefisch.
The cuticle is composed of five layers; surface coat, epicuticle, cortical zone, medial zone, and basal zone (Cox et al., 1981). Throughout development the cuticle is variable in surface protein expression, number of layers, relative thickness, and the total composition. Besides serving as the exoskeleton, the cuticle also lines the pharynx, vulva, rectum, and excretory pore (Albertson and Thomson, 1976).

The genome of *C. elegans* contains about 100 megabases and roughly 20,000 protein-coding genes (*C. elegans* sequencing consortium, 1998). Sequence conservation among other species of the genus, including *C. briggsae*, *C. remanei*, and *C. japonica*, can aid in identifying these genes in *C. elegans* (Stein et al., 2003).

The work in this thesis focuses on cell adhesion and more specifically, the cytoskeleton filament IFA-2 within the fibrous organelle (FO) adhesion complex. This work has implications for not only a basic understanding of nematode cell adhesion, and perhaps the molting process, but also may help in understanding cell adhesion in vertebrates. The intermediate filaments of the nematode hypodermis, although not orthologous, are comparable to keratins of vertebrates and the FO of *C. elegans* is functionally comparable to the hemidesmosome adhesion structure found in vertebrates (Hesse et al., 2001; Fracis and Waterston, 1991). FOs are structures central to transmitting force from the skeletal (body wall) muscles to the cuticle. The force originating from the muscle is transmitted across the intervening basal lamina, through the FOs within the hypodermis, to the outlying cuticle, which results in locomotion of the animal.
Dense bodies of *C. elegans* are adhesion complexes that serve to link muscle cells to the basal lamina by way of the actin filaments and are comparable to focal adhesions of vertebrates. The muscle proteins associated with these adhesion complexes have been conserved between *C. elegans* and vertebrates. Striated muscle of *C. elegans* is structurally comparable to the skeletal muscle of vertebrates.

Sarcomeres in vertebrates consist of the myosin thick filaments attached to the M line, and the thin actin filaments associating at the Z disk (Samarel, 2005). The *C. elegans* sarcomere also includes the myosin attachment at the M line, but dense bodies act as the homologous Z disk for anchoring the actin filaments (Cox and Hardin, 2004). Vertebrate sarcomeres are striated perpendicularly to the muscle cells long axis, while *C. elegans* sarcomeres in the body wall muscles are offset just five to seven degrees from parallel to the long axis of the muscle (Francis and Waterston, 1985). In addition, nematode muscles are mononucleated unlike the syncytial muscle fibers of vertebrates. In *C. elegans*, there are four muscle bands running the length of the animal that are separated from the epidermis by the basal lamina (Waterston, 1988). Sarcomeres are established in the two-fold stage in embryos, but increase in number and size throughout development (Krause, 1995).

Although sarcomeres and their included filaments are in an almost parallel orientation, the dense bodies are perpendicular to the filaments in muscle cells, to anchor the actin filaments to the underlying muscle cell membrane. For both vertebrates and *C. elegans* the function of the muscle is the same; to translate the force generated by the sliding of myosin heads across actin filaments into movement. In order to transmit this force outside of the muscle, muscle cells must be anchored. In vertebrates the Z disk is
anchored to the ECM by a complex known as the costamere and then muscle is linked to bones by tendons, to transmit the force into movement. (Ervasti 2003; Samarel 2005).

The dense body of C. elegans serves both functions of the Z disk and costamere, in order to anchor actin filaments to the basal lamina. The fibrous organelle adhesion complex is the analog of tendons, which links muscle to cuticle, to transmit force generated into locomotion of the worm (Cox and Hardin 2004). Similar to focal adhesions of vertebrates, integrins are the transmembrane receptors in C. elegans dense bodies, which anchor the filaments to the muscle cell membrane, and result in extracellular transmission of force.

In the C. elegans dense body, the integrins are a heterodimer composed of PAT-2/α-integrin and PAT-3/β-integrin (Gettner et al., 2003; Mercer et al., 2003). This heterodimer recruits the cytoplasmic proteins that assist in connecting the actin filament bundles to the complex, and the heterodimer is in turn anchored to the basal lamina via the ECM protein UNC-52/perlecan (Mullen et al., 1999). The other cytoplasmic proteins of the dense body are mostly homologs of vertebrate actin-binding proteins and include DEB-1/vinculin, ATN-1/α-actinin, and UNC-112/ Mig-2 (Barstead and Waterston, 1989; Barstead et al., 1991; Rogalski et al., 2000). These proteins along with others in the complex are found expressed in muscles throughout adulthood, underscoring their importance to maintaining muscle cell adhesion. In addition to a structural role, dense bodies may also be involved in signaling events, as suggested by the presence of some proteins such as UNC-112. UNC-112 may activate CDC-42 signaling through an interaction with UIG-1 (UNC-112 Interacting GEF-1) (Hikita et al., 2005). Integrins themselves have also been reported in signal transduction (Hynes et al., 2002). Therefore,
it is plausible these signaling events may control cytoskeletal rearrangements and work in concert with fibrous organelle-derived signals to coordinate assembly and maintenance of adhesion, in *C. elegans*.

The fibrous organelle (FO) is an adhesion complex that serves to transmit force from underlying basal lamina, through the hypodermis to the cuticle, in order to generate locomotion (Fig. 3). FOs are initially expressed as puncta along the anterior-posterior axis of embryos and are repositioned to the characteristic circumferential striping, during the muscle contractions of elongation (Bercher et al., 2001). The FOs are complex structures that are functionally comparable to the hemidesmosomes of vertebrates that link the IF cytoskeleton of cells to the extracellular matrix. Besides a role in tissue integrity, hemidesmosomes also have purported roles in cell migration, wound healing, and carcinoma invasion. During these events the complex has been shown to reorganize and disassemble (Litjens et al., 2006). Vertebrate hemidesmosomes consist of the proteins plectin, BPAG1 that bind IFs, as well as the α6β4 integrin and BPAG2 that anchor the complex to the basement membrane (Koster et al., 2004; Guo et al., 1995; Mercurio et al., 2001; McGrath et al., 2006).

The *C. elegans* FO consists of an apical and basal hemidesmosome-like plaque linked by IFs. IF associated proteins within the FO are believed to regulate IF incorporation and anchor them to the hemidesmosomes (Houseweart and Cleveland, 1998; Jones et al., 1998). These FOs overlie sites of compressed hypodermis that are contacted by adjacent muscle. FO formation coincides with sarcomere development (Hresko et al., 1994). The basal hemidesmosome includes the membrane receptor LET-
805, while MUP-4 and MUA-3 are membrane receptors found in the apical hemidesmosome.

LET-805, also known as myotactin, is a transmembrane protein with fibronectin repeats (Hresko et al., 1999). Besides the hypodermal expression, LET-805 is also present in the pharynx. Mutants have the characteristic paralyzed at two-fold (Pat) phenotype of animals that are unable to transmit muscle contraction during elongation, and die before hatching (Hresko et al., 1999). In addition to a role in adhesion, LET-805 may be involved with signal transduction and the organization of FOs. LET-805 staining first mimics muscle elements before assuming the typical circumferential striping of the FOs. LET-805 also contains serine-rich regions that suggest the protein may be modified by phosphorylation. It is plausible that not only does LET-805 maintain muscle attachment at the basal end of FOs, but it may be the link that signals FO assembly based on muscle contraction. The spatial patterning of FOs seems dependent on not only muscle attachment, but on muscle contractions. In both contractile and let-805 mutants, FOs are found mislocalized around the time that LET-805 would normally associate with FOs (Hresko et al., 1999).

The apical receptors MUA-3 and MUP-4 have high sequence homology that differs mostly in the cytoplasmic domains, suggesting different interactions for the receptors with other proteins or filaments within the FO (Bercher et al., 2003). Though the receptors do not have direct orthologues in vertebrates, their intracellular domains have weak similarities with the mammalian filaggrin protein that associates with IFs (Mack et al., 1993). In addition, the apical receptors likely evolved out of necessity for a second attachment site in FOs to the collagen-rich cuticle, while the basal
hemidesmosome-like plaques of FOs are more comparable to vertebrate hemidesmosomes in that a single attachment is made to the basal lamina. IFs are able to colocalize at all sites of MUA-3 expression including touch neurons, sensory dendrites, and the excretory duct (Chalfie and Sulston, 1981; Perkins et al., 1986; Nelson et al., 1983). Because of their embryonic lethality, LET-805 and MUP-4 may control FO organization early in development, while MUA-3, which is essential during larval stages, may maintain the organization.

*mup-4* mutant embryos arrest due to defects in hypodermal organization and muscle cell positions. MUP-4 is also expressed in the inner surface of the pharynx, in muscles of the anus, vulva, and intestine, and in mechanosensory neurons (Hong et al., 2001; Chalfie and Sulston, 1981).

Another member of the FO complex, VAB-10, is a plakin family member whose loss results in muscle detachment (Roper et al., 2002; Bosher et al., 2003). Plakins are known to link the cytoskeleton to junctions of membrane complexes by crosslinking actin and microtubules (Ruhrberg and Watt, 1997). Plakins also anchor IFs to membrane junctions (Green et al., 2002). VAB-10 is also a plectin orthologue and likely serves a similar role in anchoring IFs to FOs (Bosher et al., 2003). A weak mutant of the VAB-10A isoform is viable and has been used as a marker to identify other proteins essential to tissue integrity (Zahreddine et al., 2010).

PAT-12/GEI-16 is another component of the FO, which was identified in a yeast two-hybrid screen using VAB-10 as bait (Hetherington et al., 2011). The *pat-12* null leads to defects at the apical hemidesmosome, suggesting a coordinated role with VAB-10 in maintaining the apical attachments. Under electron microscopy, it appears that null
animals have an internal rupture of the hypodermis, suggesting PAT-12 may also interact directly with IFs and anchor the filaments to the hemidesmosomes. The defects lead to the Pat phenotype in embryos. (Hetherington et al., 2011). Besides the PAT-12A isoform that is expressed in hypodermal FOs and can rescue the null allele, other PAT-12 isoforms are found in the pharyngeal membranes, seam cells, spermatheca, and vulva (Hetherington et al., 2011). A diagram of the *C. elegans* fibrous organelle is shown in Figure 2.

![Figure 2 – Fibrous Organelle](image)

**Fig. 2 – Fibrous Organelle.** The fibrous organelle contains a single basal membrane receptor, myotactin (LET-805), and two apical receptors, MUA-3 and MUP-4. Intermediate filaments in the complex include IFA-2/3 and IFB-1. Accessory proteins may serve to bridge the connection between filaments and receptors. Reprinted with permission from Dr. John Plenefisch.
Genetic loss or RNAi knockdown of many of the proteins in the FO complex results in a Mua phenotype which stands for fragile muscle attachments. Mua mutants undergo progressive paralysis due to separation of muscle from the cuticle. This defect is phenotypically different from other muscle defects that either are due to dysfunctional myofibril assembly or a loss of muscle attachment to the basal lamina in the embryo (Williams and Waterston, 1994). Mua defects do not occur until the larval stages, implying that they result from a defect with slower-acting impact. Adhesion defects for Mua genes are gene-specific and defective sites range from muscle-muscle, muscle-hypodermis, to internal hypodermis rupture (Plenefisch and Hedgecock, 2000). Muscle separation correlates with stress, as muscle sites with higher levels of stress, such as the anterior head muscles and dorsal muscles near the anal depressor, tend to detach first (Plenefisch and Hedgecock, 2000). Detachment is also exacerbated by muscle contraction. Mutations in the myosin heavy chain gene, unc-54, which nearly paralyzes animals, reduces the penetrance of muscle detachment in Mua genes. Because of its nature in cell adhesion, the Mua phenotype can be used as a tool for assessing function of the FO and for identifying any other proteins that may be involved in cell adhesion. An example of a Mua animal is seen in Figure 3.
Fig. 3 – Mua phenotype. Wild-type animal (A) with cuticle (c), muscle (m), basal lamina (bl), and normally compressed hypodermis (h). Animal with recessive rh85 allele of ifa-2 (B). Integrity of hypodermis is now lost, resulting in muscle separation from cuticle and post-embryonic paralysis. Scale bars are 1μm. Reprinted with permission from Hapiak et al (2003).

The intermediate filaments (IFs) associated with the FO are the focus of this thesis. IFs are one of three major cytoskeleton filaments and are deemed “intermediate” as their diameter is between that of the smaller filament, actin, and larger microtubules. IFs are unique from the other filaments in that they spontaneously assemble. IFs represent from 1-85% of the total cellular protein, depending on cell type (Goldman, 2001). Even with this prevalence, IFs are the least understood of the cytoskeleton filaments. Roles for IFs include anchoring cell adhesions and resisting stress, while they also work in coordination with the other filaments to accomplish cell division, migration, and the transport of molecules (Carberry et al., 2009).

Loss of function in IFs has been linked to tissue fragility diseases, improper wound healing, and carcinoma invasion (Fuchs and Weber, 1994). These effects support the idea of a structural role for IFs, but also that they may serve as scaffolds to bring together molecules in the epithelium, including signaling complexes. Unlike actin and microtubules that are found in all eukaryotic cells, IFs are unique to animals (Erber et al.,
1998). This brings into question how are their roles accommodated for in other systems and just how essential are these IFs? Animals without IFs, such as *Drosophila melanogaster* seem to compensate for the lack of IFs by the use of bundles of microtubules (Hynes and Zhao, 2000). More work on IFs should help to elaborate on the roles of IFs and how they relate to the cytoskeleton as a whole.

*C. elegans* cytoplasmic IFs have been documented in the pharynx, intestine, hypodermis, and uterus. IFs in the hypodermis are associated with the FO adhesion structure in a manner similar to IFs association with the mammalian hemidesmosome adhesion structure (Hahn and Labouesse, 2001). The *C. elegans* IF gene family consists of 11 cytoplasmic intermediate filaments and a single nuclear lamin. The single *C. elegans* lamin is encoded by *lmn-1* (Riemer et al., 1993). *C. elegans* cytoplasmic IFs have the typical IF structure of a central α-helical rod domain plus the globular head and tail domains. What is unique of *C. elegans* IFs compared to vertebrates is the presence of 42 extra hydrophobic residues within a coil of the rod domain, supporting the IFs evolutionary conservation from lamins (Dodemont et al., 2004). Even though *C. elegans* cytoplasmic IFs are not orthologous to vertebrate IFs, they are still believed to behave similarly based on their comparable roles in cell adhesion. The rod domain is essential to dimerization and assembly of filaments (Herrmann et al., 2002). Little is known about the functional roles of the head and tail domains. There is evidence from mammalian systems that they may contribute to IF assembly, and that phosphorylation of these domains mediates the assembly properties (Omary et al., 2006; NO et al., 1996). SUMOylation of the tail of *C. elegans* IFB-1 appears to modulate its incorporation into the FOs (Kaminski et al., 2009). However it is also likely these domains, given their
exposed position in the mature IFs, will associate with IF accessory proteins, including proteins within vertebrate hemidesmosomes and the nematode FO.

With alternative splicing and differential promoter expression, there are a total of 15 unique cytoplasmic IF proteins encoded by the 11 cytoplasmic IF genes (Woo et al., 2004). The 11 cytoplasmic IF genes identified in *C. elegans*: *ifa-1*-4, *ifb-1*-2, *ifc-1*-2, *ifd-1*-2, and *ife-1*, all are more homologous to the nuclear lamins, from which they probably evolved, rather than to the vertebrate cytoplasmic IFs (Fridkin et al., 2004). So at least on a physical level the *C. elegans* IFs share more relation to the nuclear lamins than to the other cytoplasmic IFs of vertebrates, though as in the case of the vertebrates, the *C. elegans* IFs display redundancy (Woo et al., 2004). Mutations in many of the genes result in weak phenotypes that are enhanced in simultaneous mutation of multiple genes. While some IFs can compensate for loss of others, a subset of IFs are essential and required for viability.

Because of the ability to do RNAi in *C. elegans* and localize proteins by fluorescence using the green fluorescent protein (GFP), characteristics for these different IFs can be easily ascertained. Phenotypes resulting from IF depletion by RNAi include: for *ifa-1*: early larval lethality and a swollen intestine (Karabinos et al., 2001). For *ifa-2*: early larval lethality and paralysis as a result of body wall muscle displacement (Karabinos et al., 2001). RNAi of *ifa-3* results in late embryonic lethality and hypodermal detachment from cuticle (Karabinos et al., 2001). RNAi of *ifb-1* leads to late embryonic lethality with an abnormal hypodermis and IFC-2 depleted animals exhibited a milder, dumpy phenotype (Karabinos et al., 2003; Karabinos et al., 2004). All other IFs display no phenotype individually.
Multiple IFs have been found to be expressed within the same tissues, suggesting cooperation and validating redundancy between the different IFs. Unique tissue expression for some IFs also suggests individual roles for specific IFs. For example, IFA-2, IFA-3, and IFB-1 are all localized to the FOs of the hypodermis while IFB-1 is also found in amphid sensory neurons (Hapiak et al., 2003; Karabinos et al., 2001; Karabinos et al., 2003). Expression of IFs can be separated into two groups: expression in a tissue along with IFB-1, or expression within the intestine, where IFB-1 is not found. These differences in tissue expression could explain why the IF genes are temporally regulated throughout development. *ifb-1* mRNA levels are constant throughout development, while *ifa-2* is up-regulated in larvae and *ifa-3* is down-regulated in adults (Karabinos et al., 2002). IF genes also vary in their importance, at least based on their corresponding lethality. The four essential IFs include *ifa-(1-3)* and *ifb-1*, while the other IFs only result in mild, viable phenotypes (Karabinos et al., 2001). While *ifa-1/2* result in larval lethality, *ifa-3* and *ifb-1* result in embryonic lethality, suggesting a dependence of certain IFs at certain developmental stages, in order to establish and maintain structural integrity. A diagram of IF maturation may be seen in Figure 4.
Fig. 4 – Maturation of filaments. Dimerization partners assemble in sequential order through association of the rod domain. Exposed head and tail domains in mature filaments likely interact with accessory proteins within the fibrous organelle. Reprinted with permission from Dr. John Plenefisch.

IFs can be modified by post-translational modifications, including phosphorylation and SUMOylation that affect their stability and assembly.

Phosphorylation of keratins has long been known to affect their assembly state (NO et al., 1996). The vertebrate p21-Activated Kinase (PAK-1) phosphorylates the IF protein vimentin, and the C. elegans orthologue PAK-1 also leads to IF phosphorylation, as detected by an antibody in 2-d gel analysis (Zhang et al., 2011). SUMOylation is also believed to affect IF dimerization and maturation. A C. elegans orthologue of SUMO, the small ubiquitin-related modifier (SMO-1), is required for IFB-1A localization to FOs (Kaminski et al., 2009). smo-1 mutants have a decreased exchange rate of IFB-1A into FOs from a soluble pool, and RNAi of smo-1 abolished this pool of IFB-1A. IFA-2 was
also found as a putative target of SMO-1 (Kaminski et al., 2009). Therefore SUMOylation may be used to sequester a pool of IFs that can be called upon by the FO when attachments are weakened or nascent filaments are being formed.

IFA-2, originally termed “MUA-6”, is an essential intermediate filament whose loss leads to muscle detachment and paralysis (Hapiak et al., 2003). IFA-2 is one of four IFs found to localize to FOs in the hypodermis, but is also expressed in the vulva, pharynx, and cells of the amphid sensory neurons along with some unidentified neurons in the nerve cord (Hapiak et al., 2003; Karabinos et al., 2002, 2003). While the protein is expressed in embryos, it is the only IF in FOs not required for embryonic development, but is essential at the larval stages. The cell adhesion defect in ifa-2 Mua animals is an internal hypodermal rupture that results in the muscle detachment (Hapiak et al., 2003). Other IFs can compensate for loss of IFA-2 until the L1-L2 stages that correlate with increased strength of contractions (Hapiak et al., 2003).

The work presented here focuses on exploring roles for the head and tail domains of IFA-2. My hypothesis was that the head and tail domains of IFA-2 are able to associate with the membrane receptors of the FO, in order to anchor the complex. IFA-2 variants tagged with GFP were expressed in animals, to look at functional characteristics of the different domains. In vitro work using the yeast two-hybrid system was performed to identify any interactions the head and tail domains may participate in, with regards to the context of the FO. Here in this thesis I show that the head domain alone can localize to FOs, suggesting an interaction with a protein of the complex, and that the tail domain alone cannot be localized to FOs, suggesting a dependence upon the other domains of IFA-2 for its function. Furthermore, I show that he head and tail can directly interact in...
vitro, and that the head domain potentially interacts with a wide variety of functional classes of proteins.
2. Materials and Methods

Worm Maintenance and Strains

*C. elegans* were grown and maintained using standard methods. Experiments were performed at 16°C unless otherwise noted. All other strains whose origin is not cited below were obtained from the Caenorhabditis Genetics Center (CGC). Standard genetic methods were used (Lewis and Flemming, 1995).

Bacterial Transformation

NEB 5-alpha competent *E. coli* cells (New England BioLabs, Ipswich, MA) were used for transformation and the supplier’s high efficiency protocol was followed. Cells were thawed on ice and 50μl were used for each transformation. 1-5μl of DNA was added to cells and tubes were flicked to mix. The mixture was put on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and then put back on ice for 5 minutes. 950μl room temp SOC was added and solutions were incubated at 37°C for 1 hr with shaking. 100μl and 900μl portions of the transformation were spread onto LB plates containing the appropriate selective antibiotic. Plates were incubated overnight at 37°C for induction of colonies.
Plasmid Miniprep

Unless noted, the alkaline lysis method was used when isolating DNA (Birnboim and Doly, 1979). Bacterial colonies were cultured overnight at 37 °C in 3.0mls of LB media and the appropriate antibiotic. 1.5mls of cultures was transferred to microcentrifuge tubes and were spun at 12,000 x g for 1 minute in a microcentrifuge (Fisher, Waltham, MA) then supernatants were discarded. 125μl GTE was added to resuspend the pellet followed by 200μl NaOH/SDS. The solution was incubated at room temperature for 5 minutes, and then 185μl 5M potassium acetate was mixed and incubated on ice for 5 minutes. The samples were spun at 12,000 x g for 10 minutes and then supernatants were transferred to tubes with 1ml of 100% EtOH. Samples were centrifuged at 12,000 x g for 10 minutes, washed with 300μl 70% EtOH for 3 minutes, then pellets were allowed to dry overnight. Samples were resuspended with either dH2O or TE for storage.

Miniprep for Microinjections

The DNA prepared for microinjection of worms was obtained by a variation of the alkaline lysis method. After pelleting the cell debris, 50μl of 5% CTAB was added to the transferred supernatant and incubated at room temp for 10 minutes. Samples were centrifuged at 12,000 x g for 10 minutes, the supernatant was decanted, and 600μl of 1.2M NH₄OAc along with 1mL of EtOH was added to pellets. After being centrifuged at 12,000 x g for 10 minutes, samples were washed with 500μl EtOH for 3 minutes then dried overnight.
Polymerase chain reaction (PCR) was performed using Phusion High-Fidelity PCR Master Mix (New England BioLabs, Ipswich, MA) and their suggestions were considered when designing the setup. PCR reactions were done in 20μl volumes. This included 1μl of Primer A, 1μl of Primer B, 10μl Phusion Master Mix, 1μl of DNA template, then dH2O added to a total volume of 20μl. For some templates, 0.6μl DMSO needed to be added to the mix to improve amplification yields. Samples were run in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA) with a program tailored to the specific reaction. Typically this included an initial denaturation of 98°C for 30s. Next denaturation, annealing, and extension steps were performed at 98°C for 15s, X°C for 30s, and 72°C for 60s/1kb of product, respectively. $X^\circ = Tm + 5^\circ C$ of the lowest Tm primer and the set of steps were cycled 30 times. Finally an extension at 72°C for 5min was done before holding samples at 4°C until they could be run on an agarose gel for verification and extraction of predicted bands.

**Agarose Gel DNA Extraction**

To extract DNA after being run on an agarose gel, the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) was used. After electrophoresis of the gel, the DNA band of interest was excised and placed in a tube with 10μl Membrane Binding Solution per 10mg of gel slice. The gel was dissolved by heating at 65°C for about 10min. The dissolved mixture was transferred to a minicolumn assembly consisting of a SV Minicolumn inserted into a collection tube. After incubation for 1min the mixture was filtered by centrifugation at 12,000 x g for 1min. Flowthrough was discarded and the
minicolumn was reinserted into the collection tube. The sample was washed first with 700μl Membrane Wash Solution and centrifuged at 12,000 x g for 1min followed by a 500μl wash for 5min. The empty minicolumn assembly was then spun for 1min with the centrifuge lid off for evaporation of any residual ethanol from the Membrane Wash Solution. The minicolumn was then transferred to a new microcentrifuge tube where the DNA was eluted by adding 20μl dH2O onto the filter, incubating for 1min, and centrifuging at 12,000 x g for 1min. The flowthrough would then contain all DNA eluted through the minicolumn.

Microinjection

To generate worms expressing specific fluorescently-tagged proteins of interest, germline microinjection was used (Mello and Fire, 1995). N2 hermaphrodite young adults were injected with DNA containing GFP-tagged reporter constructs, into the syncytial germline cytoplasm of both distal gonad arms. The injection mix typically included 50ng/μl GFP-tagged construct, 50ng/μl rol-6 co-injection marker, 50ng/μl herring sperm carrier DNA in TE. Expression of the rol-6 marker results in animals with a roller phenotype which helps to indicate a successful injection. In some cases, the carrier DNA was not included. Worms were immobilized on 2% agarose pads in the center of microscope cover glass slides (35x50mm) (Fisher, Waltham, MA). Halocarbon oil was placed on the injection pads to prevent dehydration of transferred worms. After injection the worms were washed with M9 before being transferred back to nematode growth media (NGM) plates. Needles used for injection were capillary tubing with catalog # 30-30-0 (FHC Inc., Bowdoin, ME) prepared by a micropipette puller.
Injections were performed on a Nikon Eclipse TE 2000-S inverted microscope (Nikon Instruments Inc., Melville, NY). After the injection mix was loaded into the pulled needle, the tip of the needle was broken on the side of the agarose on the injection pad to allow for flow of the mix into the worm when pressure is applied. When the setup was ready for injections, a young adult was transferred to the oil on the injection pad and then placed on the stage of the microscope. The tip of the needle was brought level with the cuticle adjacent to the target of the gonad and the needle pierced the worm by a slight tapping of the stage. After pressure was applied and the injection mix filled up the gonad, the needle was retracted and the process repeated in the other arm of the gonad. To recover the animal, M9 was added to wash off the remaining oil and allow for rehydration of the animal, before being transferred to a fresh NGM plate and allowed to propagate. After incubation in 20°C for about 3 days, the plate was screened for any F1 animals possessing the roller phenotype. Individual F1 rollers were transferred to new plates and observed for any progeny that also roll, indicating transmission of the construct and a stable line.

GFP Reporter Constructs

The LET-805 cytoplasmic domain::GFP reporter construct driven by the dpy-18 hypodermal promoter was generated as follows: The let-805 cytoplasmic domain sequence was amplified by PCR off of genomic N2 DNA using let805U and let805R primers that contain engineered MluI and BglII restriction sites, respectively. The complete sequence of the promoter-less pat-3 transmembrane domain::GFP vector L4058 was amplified by PCR using L4058F and L4058R primers containing restriction sites of
MluI and BglII to generate an in-frame insertion site for *let-805* sequence between the *pat-3tm* and *gfp* sequences. The amplified *let-805* was then ligated in frame by digesting both insert and vector with MluI and BglII, mixing the insert and vector DNA in a 2:1 ratio, and ligating with Quick Ligase (New England BioLabs, Ipswich, MA) using the manufacturer’s protocol. The ligation was transformed into NEB 5-alpha competent *E. coli* cells and recovered by plasmid miniprep, as previously described. Colonies containing properly-ligated samples were identified by restriction digest to verify presence of the insert in the proper orientation. This *let-805::L4058* construct was designated “pKW10”. The *dyp-18* promoter was amplified by PCR from genomic N2 DNA using PstIFd18 and EcoRVRd18 primers, with engineered PstI and EcoRV restriction sites. The *dpy-18* product was then digested and ligated into the promoter insertion multiple cloning site of the pKW10 construct cut with PstI and SmaI. The final product was designated “pKW11” and is 10.2kb in length including 4.7kb of L4058, 2kb of *dpy-18*, 3kb of *let-805* and 0.5kb for the *let-805* transmembrane domain. The structure of pKW11 was confirmed by sequencing.

In order to make double-labeled transgenic animals, an *ifa-2::rfp* construct was generated. The goal was to take the *ifa-2* portion of an *ifa-2::gfp* clone, made by previous lab member Hallie Baucher, and ligate it to the *rfp* portion of the tagRFP in pPD117-01 clone, kindly provided by Dr. Robert Steven. For PCR of *ifa-2* we used GFPrepR (MluI) and GFPrepIF (EcoRV) primers to isolate an 8kb piece from the *ifa-2::gfp* clone. For *rfp* the PCR used RFPinsF (MluI) and RFPinsR (EcoRV) primers to isolate an 870bp portion from the tagRFP in pPD117-01 clone. After PCR products were obtained, samples were
digested with the respective enzymes, ligated, and verified by restriction digest and sequencing the joints.

Yeast Two-Hybrid Constructs

For beginning the Y2H work we wanted to test the tail-only portion of IFA-2 (IFA-2T) as a bait against MUA-3 and LET-805 as preys. For ifa-2t, primers were IFA-2F (EcoRI) and IFA-2R (XhoI). Let-805F (EcoRI) and Let-805R (Sall) were primers for let-805 and Mua3F (BglII) and Mua3R (Sall) were primers for mua-3. The ifa-2t PCR product was amplified from the ifa-2t::gfp construct generated previously, while let-805 came from the pKW11 construct previously described and mua-3 from a mua-3::gfp construct made by a previous lab member (Isaac Perry, Honors Thesis, 2011). These products were digested with the respective enzymes and ligated to the pEG202 bait plasmid cut with BamHI and XhoI, and the modified pJG4-5 prey plasmid cut with BglII and XhoI. All six Y2H constructs (mua-3::pEG202, mua-3::pJG4-5, let-805::pEG202, let-805::pJG4-5, ifa-2t::pEG202, and ifa-2t::pJG4-5) were verified by sequencing.

pEG202 bait constructs were verified by sequencing using PET-1F and PET-1R primers, while pJG4-5 prey constructs were verified with LCF-1 and LCR-1 primers. All primers for sequencing read into site of insertion. After the initial Y2H screen we decided to also look at a possible interaction between the head domain only of IFA-2 (IFA-2H) and either LET-805 or MUA-3. Primers designed for IFA-2H include HOF that introduced an EcoRI site and HOR that introduced a XhoI site into the 222bp amplified ifa-2h fragment. The ifa-2h PCR product was ligated into bait and prey plasmids and verified as described previously.
After the library screening with IFA-2H as bait, the transformants were miniprepped and then PCRed to amplify the library inserts found to interact with IFA-2H. The primers used for PCR were RS480 and RS481. To identify the library inserts, the PCR products were then sequenced using RS480 which reads into the 5’ end of the pB42AD library prey plasmid. Sequencing was conducted by the sequencing core at The University of Michigan. Miniprep, PCR, and sequencing was carried out in the same fashion for library transformants using IFA-2T as the bait.

To further investigate one of the proteins picked up in the library screen, K04G7.1, I had to design primers for amplification and ligation into the pPD95.75 GFP expression plasmid (gift of A. Fire, Stanford University). To PCR K04G7.1 from genomic N2 DNA I used the primers K04F that introduced a XbaI site and K04R with an XmaI site. After K04G7.1 was amplified, it was digested with the respective enzymes and ligated to the multiple cloning site of pPD95.75 cut with the same enzymes. Primers used for generating Y2H constructs and GFP expression constructs may be seen in Table 1.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Site</th>
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<tr>
<td>EcoRVRd18</td>
<td>GGGATATCTGTCTGAAAAATAACTTCTTAT</td>
<td>EcoRV</td>
</tr>
<tr>
<td>GFPrepR</td>
<td>GGACGCCTATTCTTTCTACCCGTACCCCTCC</td>
<td>M11</td>
</tr>
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<td>GFPrepLF</td>
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<td>XhoI</td>
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<td>XhoI</td>
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<tr>
<td>LCR-1</td>
<td>CGACCAACCTTGATTTGGAGAC</td>
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<td>RfpinsR</td>
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<td>EcoRV</td>
</tr>
</tbody>
</table>

**Table 1 – Primers used in the study.** Primers used for cloning and sequencing are listed. Restriction sites that were engineered into 5’ ends for cloning the amplified DNA are listed when appropriate.
Yeast Plasmid Miniprep

Yeast minipreps were performed using Zymoprep I™ Yeast Plasmid Minipreparation Kit from Zymo Research (Irvine, CA). Individual yeast colonies were picked and cultured overnight at 30°C in YPD or selective media. Cultures were centrifuged at 12,000 x g for 1 minute. Supernatants were discarded and 150μl of Solution 1 was added to pellets, followed by 2μl of Zymolyase™. Following incubation at 37°C for 1 hr, 150μl each of Solution 2 and Solution 3 was added. Samples were centrifuged at 12,000 x g for 2 minutes and transferred to new tubes with 400μl isopropanol. Samples were centrifuged at 12,000 x g for 8 minutes, supernatants were decanted, and pellets allowed to dry.

Small-scale Yeast Transformation

To transform yeast with a single plasmid a protocol was adopted from Clontech (Mountain View, CA). For preparation of competent yeast cells, we started off with EGY48 cells, already containing the pSH18-34 reporter, which were generously provided by Dr. Scott Leisner. pSH18-34 provides a blue color in x-gal when activated and allows for uracil selection. A clump of EGY48 + pSH18-34 cells was incubated in 3ml of –U dropout supplement at 30°C with shaking for 8-12hrs. Based on the observed density, 50-150μl of culture was transferred to 50ml of fresh media and incubated again, depending on if the bait or prey was to be transformed. For a bait transformation, the density was not important and cells were incubated overnight. For a prey transformation using yeast already containing the bait plasmid, cells were incubated until the OD_{600} was
0.8-1.0. In either case, cultures were transferred to 50ml conical tubes and centrifuged for 5min at 4°C. Supernatant was discarded and pellets were resuspended with 30mL deionized water and spun again for 5min at room temp. Supernatant was again discarded and pellets were now resuspended in 750μl of 1.1xTE/LiAc. Solutions were transferred to a 1.5ml centrifuge tube, centrifuged for 15s and decanted. Samples were finally resuspended with 600μl 1.1xTE/LiAc and stored on ice until ready for transformation.

To begin the transformation 200ng of either bait or prey plasmid DNA was added to 5μl of sheared, salmon sperm (carrier) DNA (Trevigen, Gaithersburg, MD) denatured by boiling for 10min. The bait plasmid used was pEG202, allowing for histidine selection, and the prey was pJG4-5 modified with additional restriction sites, that allows for tryptophan selection. Both plasmids were constructed with DNA of the proteins of interest cloned into the multiple cloning sites. 50μl of competent yeast cells followed by 500μl PEG/LiAc was added and samples were incubated at 30°C for 30min. 25μl DMSO was added and then samples were incubated at 42°C for 15min, with vortexing every 5min. Cells were pelleted by centrifugation for 15s, supernatants decanted, and pellets resuspended with either 150μl of appropriate selective media for bait transformation or 300μl for prey transformation. Solutions were incubated at 30°C for 1hr and then spread on the appropriate selection plate. Plates were incubated at 30°C for 3-5 days for induction of colonies.

Transformants plated and incubated on –U/-H media plates yielded a confluency of about 500 colonies per plate. Colonies were restreaked onto –U/-H plates to maintain a stock of bait-transformed yeast to be used for prey transformations. Next, the various prey transformations were performed in conditions as similar as possible to the bait
transformations. The only exceptions were that the bait-transformed yeast was used as
the starting cells, the pJG4-5 constructs were now being transformed into yeast, and
transformants were now plated on –U/-H/-T and –U/-H/-T/-L/gal/raf/x-gal plates to check
for transformation efficiency and ability of the plasmids to induce activation of reporter
genes respectively.

Large-scale Yeast Transformation (Library Screen)

To begin, EGY48 cells already containing the pSH18-34 reporter plasmid and
IFA-2H::pEG202 bait plasmid, were inoculated into 1mL of –U/-H media. This solution
was immediately transferred to 150mL –U/-H media and grown overnight at 30°C to a
stationary phase where OD$_{600}$ > 1.5. Then a volume of this culture was transferred to 1L
of YPD, enough to give an OD$_{600}$ = 0.2-0.3. Again the culture was incubated for about
3hrs, until OD$_{600}$ = 0.5. The cells were divided among four, 250ml plastic bottles
(Nalgene, Rochester, NY) and centrifuged at 1,500 x g for 5min. Supernatants were
decanted and pellets washed by resuspending with 125ml dH$_2$O and then centrifuged
again at 1,500 x g for 5min and supernatants discarded. The four pellets were
resuspended with 2ml of 1xTE/LiAc each and then pooled together in the same bottle.
Then 0.1mg cDNA library, 20mg sheared, salmon sperm (carrier) DNA, and 60ml
PEG/LiAc was added to the cells and incubated at 30°C for 30min. The cDNA library
(Origene, Rockville, MD) is in the pJG4-5 plasmid and was generously provided by Dr.
Robert Steven group. Carrier DNA was denatured by boiling for 20min and chilled
quickly prior to transformation. Next, 7ml DMSO was added and the solution was heat
shocked at 42°C for 15min. Cells were centrifuged at 1,500 x g for 5min, supernatant
decanted, and the pellet resuspended by 10ml of 1xTE. For plating of the suspension, 100μl of either a 1:1,000, 1:100, or 1:10 dilution was spread on –U/-H/-T plates for cotransformation efficiency controls. A 1:100 dilution was also spread on –U/-H and –U/-T plates for controls of single-plasmid transformation efficiency. The remaining suspension was spread onto 40 –U/-H/-T plates, using 200μl/plate and two –U/-H/-T plates using 500μl and 1mL. Plates were incubated at 30°C for 3-5 days for induction of colonies. 7 of the 40 –U/-H/-T plates spread with 200μl of the suspension were contaminated and were discarded to prevent contamination of the glycerol stock of transformants. The –U/-H control plate was covered with colonies, verifying the stock of cells used in the transformation contained the bait plasmid. The –U/-T control plate only had contaminants, suggesting the transformation of the library prey plasmid was inefficient. The three –U/-H/-T control plates were also contaminated, but colonies were counted to get an estimate of the colony forming units (cfu), in order to calculate titer of the stock. I determined the dilution factor was 1:10 and that the titer of the glycerol stock would be 100,000 cfu/μl. To harvest colonies, the remaining 33 standard –U/-H/-T plates were put in 4°C for 4hrs to harden. 5ml of 1xTE pH 7.0 was added to each plate and colonies were scraped into 50ml conical tubes. The tubes were centrifuged at 1,500 x g for 5min, supernatant decanted, and pellets were resuspended with 50mL 1xTE. To make a glycerol stock, 50ml 65% glycerol/MgSO$_4$ was added to the resuspension and 1ml aliquots were stored at -80°C. To obtain 2.0x10$^6$ cfu/plate, 20μl of the glycerol stock was spread onto –U/-H/-T/-L/gal/raf/x-gal selection plates, to screen for protein-protein interactions detected by the presence of blue growth. This was done for 51 plates that were incubated at 30°C for 6 days. All the plates showed bacterial contamination and
subsequent attempts at plating the stock also led to bacterial contamination, suggesting the glycerol stock was actually contaminated. To obtain a sterile stock of library transformants, the two –U/-H/-T plates that had been plated with a higher volume of resuspension were used to prepare a new stock, as previously described. This fresh stock did not lead to contamination when spread onto –U/-H/-T/-L/gal/raf/x-gal plates and allowed us to select 70 individual colonies that had blue growth. Individual blue colonies were then restreaked onto –U/-H/-T plates and incubated overnight. A single colony was then restreaked onto –U/-H/-T plates twice again for segregation of multiple libraries within a single colony. To look for retention of β-gal activity, the colonies were restreaked onto –U/-H/-T/-L/gal/raf/x-gal plates again and incubated for 6 days. All populations that maintain β-gal activity by yielding blue growth were then stored on –U/-H/-T plates at 4°C.

Of the 70 colonies selected, 16 were considered false positives as they did not retain the blue growth. Remaining samples were miniprepped and PCRed, as previously described, to obtain the library inserts found to interact with IFA-2H. PCR samples were sent to the DNA Sequencing Core at The University of Michigan to be sequenced. The BLAST search engine at WormBase was used to identify the sequences. Only two of the remaining 54 samples were unable to be identified either due to failure to PCR samples after three attempts, or because samples could not be sequenced by the DNA Sequencing Core. Examples of activity may be seen in Figure 5.
Fig. 5. - Example of β-gal activity of sample yeast clones from the Y2H library screening. Selection plate is –U/-H/-T/-L/gal/raf/x-gal used for selecting transformants of both bait and prey plasmids that indicates β-gal activity by induction of blue growth. Yeast population 1) is transformant deemed as false positive since it was unable to retain blue growth when restreaked onto selection plate. 2) Is a positive control provided by Dr. Robert Steven. Yeast is a result of a previous Y2H library screening where UNC-73 has a positive interaction with a protein encoded by the library (note regions of blue growth). 3) Is a Y2H clone using IFA-2H as the bait and library as prey. Blue growth is comparable to positive control of 2)
Some samples yielded multiple PCR products, but there was no established routine for which bands were sequenced. A maximum of three bands were sent out for sequencing, but typically only two bands were sequenced when multiple bands were present in the PCR. The bands that were to be further analyzed were the brightest bands within the 0.2-3.6kb size range when run on an agarose gel. This is the size range of inserts encoded by the library, according to the manufacturer. While there may have been some non-specific PCR bands amplified, higher-intensity bands were considered to be true amplification by primers (RS480 and RS481, Table 1) specifically designed to amplify the library insert as the template.

More glycerol stock was spread onto selection plates for identification of 86 new blue colonies that were selected for further analysis. Of these, 30 proved to be false positives, again by failure to retain blue growth, and 3 could not be identified, leading to 53 more samples identified. In attempt to identify a varied set of interactors of IFA-2H, I decided to go back to the original glycerol stock of library transformed yeast that had been contaminated. We worked around the contamination by plating the stock on the –U/-H/-T/-L/gal/raf/x-gal selection plates and then selecting only blue colonies that were isolated from the contamination. 83 more colonies were picked for further analysis and these colonies appeared to be a darker blue than previous colonies, suggesting these may have a stronger interaction with IFA-2H than the first 156 samples investigated. Of the 83 colonies, six were deemed false positives, four led to contamination when restreaked, and one was unable to be identified. In total, the 177 unique yeast populations that were
successfully analyzed led to 206 sequences being identified, because of additional inserts analyzed for some samples.

The transformation of IFA-2T with the cDNA library prey plasmid was performed identically to the previous library transformation. The only difference was that 47 –U/-H/-T plates were used for plating the resuspension. This transformation appeared less efficient than the first one as the –U/-T plate had only a few colonies and all –U/-H/-T dilution control plates had no colonies. Though the –U/-H plate was confluent and standard –U/-H/-T plates had about 10 colonies each, but also contained contamination. Because the small number of colonies did not warrant making a glycerol stock of this transformation, colonies that were not contaminated were picked individually and streaked onto –U/-H/-T/-L/gal/raf/x-gal plates to screen for blue growth. This was done for 28 unique colonies which were further confirmed for β-gal activity by restreaking onto standard –U/-H/-T plates three times and then back onto –U/-H/-T/-L/gal/raf/x-gal plates in attempt to segregate multiple copies of library inserts that may have been present in the populations. Only two populations of yeast were determined to be false positives as they did not retain blue growth when restreaked onto the final x-gal plate. The rest were miniprepped, PCRd, and sequenced, as previously described, to obtain identities of the library inserts found to interact with IFA-2T. 13 of the remaining 26 samples could not be identified. The remaining 13 samples were sequenced and because of a single occurrence of multiple inserts in a sample, 14 sequences were identified from the IFA-2T library screen.
β-galactosidase Assay

The β-gal assay was used to quantify the interaction between yeast two-hybrid (Y2H) partners. Yeast colonies tested came from –U/-H/-T/-L plates containing yeast transformed with both bait and prey plasmids. Colonies from the –U/-H/-T plate of the pEG202::pJG4-5 transformation were used as a negative control. Yeast colonies were cultured in 3mL of –U/-H/-T/-L/gal/raf dropout media (Clontech Labs, Mountain View, CA) at 30°C overnight with shaking. 1ml of samples was used to obtain an absorbance reading at OD$_{595}$ while another 1ml was centrifuged at 12,000 x g and supernatant was decanted. 800μl of Buffer Z, 2 drops of 0.1% SDS and 3 drops of chloroform was added and vortexed in samples for 60s. 1L of Buffer Z consists of 16g Na$_2$HPO$_4$●7H$_2$O, 5.50g NaH$_2$PO$_4$●H$_2$O, 0.75g KCl, and 0.246g MgSO$_4$●7H$_2$O, with 2.7ml of 2-mercaptoethanol added prior to use. 200μl Buffer Z with 4mg/ml ONPG was added to samples and incubated at 30°C and timed, until samples reached a sufficient yellow color. Upon color transformation 500μl of 1M Na$_2$CO$_3$ was added to samples to stop the reaction and obtain the final time within the reaction took place. Endpoints for the reaction ranged from 1 to 25 minutes. Samples were centrifuged at 12,000 x g for 10min and supernatant was measured for absorbance at OD$_{420}$. To quantify the interaction between the two proteins, the absorbance readings from the samples and amount of time taken to turn yellow were used in the following equation where β-gal is in arbitrary units: β-gal = 1000 x volume of sample (ml) x OD$_{420}$ / time of reaction (min) x OD$_{595}$. 

36
3. Results

Expression of FO Components

Previous work has shown that the head domain of IFA-2 is not required for normal filament function, but that the tail domain is essential (Kristen Williams, Ph.D. Thesis 2008, Hallie Baucher, Honors Thesis, 2010). While IFA-2 lacking the tail domain (IFA-2ΔT) could not rescue a null allele of ifa-2, the mutant can still be incorporated into FOs. This incorporation however is qualitatively lower than that of the full-length IFA-2 and IFA-2 lacking the head domain (IFA-2ΔH), as observed by the fluorescence intensity of GFP-tagged constructs. This suggested that the tail domain may interact directly with FO components, and consequently it might be possible to observe a GFP-tagged tail localizing to FOs. The head domain was dispensable for IFA-2 function as it was not required for rescue of the null allele and had fluorescence intensity comparable to that of the full-length IFA-2. It is possible that head domains of other filaments within the FO, IFA-3 and IFB-1, serve a redundant function of the IFA-2 head domain and are able to compensate for its loss. So we cannot exclude the possibility that the head domain of IFA-2 may also bind directly to the FO. The rod domain of IFs have been established as being essential for IF dimerization (Hatzfeld and Weber, 1990). Some evidence suggests the tail domain contributes to the efficiency of IFB-1 incorporation into filaments (Kaminski et al., 2009). Therefore it is possible both the head and tail domains of IFA-2 play important roles in enhancing IF assembly. To test these predictions, GFP-tagged
constructs (provided by Dr. John Plenefisch) that included only the rod domain from amino acids 74-438 (IFA-2R::GFP), only the head domain amino acids from 1-74 (IFA-2H::GFP), or the tail domain amino acids from 438-581 (IFA-2T::GFP) of IFA-2 were expressed by microinjection into wild-type worms. After microinjection of each, accompanied with the rol-6 marker into N2 animals, transgenic lines were observed under epifluorescence by the compound scope.

To determine the effects of the introduced expression constructs on the health of the animals, an assay for Mua animals was performed. Mua is a fragile muscle attachment phenotype common to mutations in IFA-2 in which the muscle is no longer attached to the hypodermal tissue. The number of Mua animals and their corresponding life stages were noted. A relationship between Mua as a result of the expression construct was determined by noting Mua animals that also had GFP expression. This allowed us to determine if animals were sick as a result of exogenous protein expression and at which stage this may be affecting the animal.

IFA-2R was found to properly localize to FOs (Fig.6), but only in 58% (35/60) of animals. In 3% (2/60) of animals the FO patterning was patchy, appearing as unzippered portions of striping (Fig. 7). Furthermore, 22% (13/60) of animals were found to be Mua.

IFA-2H localized to FOs (Fig. 8), but only during larval stages, at a frequency of 67% (44/65) of animals observed. Expression in adults was restricted to the hypodermal ridges. In 18% (23/125) of the IFA-2H::GFP carrying animals where FO localization was present, a Mua phenotype was observed. The Mua phenotype also correlated with higher levels of transgene expression at the FO (Fig. 9) as 95% (19/20) of rolling Mua animals showed relatively bright expression within FOs compared to 22% (5/23) of rolling non-
Mua animals of similar stages that had incorporation at the FO, but at lower intensities than that of Mua animals.

**Fig. 6 – Rod domain of IFA-2 localizes to FOs.** IFA-2::GFP in *ifa-2(nc16)* null animal (A) and IFA-2R::GFP in wt animal (B). Full-length construct shows typical striping pattern (arrows) common to native IFs. IFA-2R::GFP also localizes to FOs, but at a lower intensity than wt. Scale bars are 10μm and (A) was reprinted with permission from Dr. John Plenefisch.

**Fig. 7 – IFA-2R::GFP Phenotype.** Photomicrograph of an animal expressing the rod-only portion of IFA-2 showing a defect in FO incorporation. Circumferential striping is not intact throughout length of animal (arrows). Scale bar is 20μm.
Fig. 8 - IFA-2H::GFP Expression. Photomicrograph of an animal expressing the head domain alone of IFA-2 showing localization of head to FOs. Scale bar is 10μm

Fig. 9 - IFA-2H results in Mua phenotype. Photomicrograph of an animal expressing IFA-2H::GFP (A) has muscle detachment, likely due to overexpression of the construct. Scale bar is 20μm. (B) is an enlargement of the area boxed in (A), showing muscle displaced from the cuticle (arrows). Scale bar is 10μm
Finally, the tail domain alone was insufficient for FO localization (Fig. 10).

Although IFA-2T::GFP array-carrying animals showed GFP expression in the hypodermal ridges, it was never observed within the FOs (55 animals). No Mua animals were observed for this line. In conclusion, we have confirmed that rod domain of IFA-2 is sufficient for incorporation into FOs. The head domain appears to have a direct interaction with the FO, as it can properly localize when expressed alone, while the tail cannot localize on its own and may require the rod domain for proper localization and interaction with the FO.

![Image](image.png)

**Fig. 10 – IFA-2T::GFP does not localize to FOs.** Photomicrograph of an animal expressing the tail domain alone of IFA-2. Unlike the head domain, the tail of IFA-2 cannot localize to FOs on its own. IFA-2T::GFP was restricted to epidermal ridges in all animals observed. Scale bar is 10μm.
Interaction Between FO Components

During development, the positioning of IFA-2 and the FO receptor proteins LET-805, MUA-3, and MUP-4 are tightly coordinated. To test if IFA-2 positioning can determine where FO receptors are positioned, I examined whether LET-805 could accumulate at sites of ectopic epidermal IFA-2 expression. A translational reporter was constructed for the basal membrane FO receptor LET-805, fused to GFP. This construct was driven by a *dpy-18* hypodermal promoter to ensure epidermal expression. LET-805::GFP was found localized to FOs at all stages of development (Fig. 11), as previously reported for the protein.

**Fig. 11 – LET-805::GFP Localizes to FOs.** Photomicrograph of an animal expressing LET-805::GFP showing localization to FOs. Scale bar is 10μm.

Since the LET-805 construct used GFP as the reporter, I combined it with an IFA-2::RFP reporter to distinguish the two proteins in animals transgenic for both. The IFA-
2::RFP expression pattern is indistinguishable from the original IFA-2::GFP (Fig. 12). IFA-2::RFP expression also results in ectopic aggregates of IFA-2 in the epidermis, which are common to expression of fluorescently-labeled IFs.

![Photomicrograph of an animal expressing IFA-2::RFP showing localization indistinguishable from IFA-2::GFP construct. Scale bar is 10μm.](image)

**Fig. 12 - IFA-2::RFP expression.** Photomicrograph of an animal expressing IFA-2::RFP showing localization indistinguishable from IFA-2::GFP construct. Scale bar is 10μm.

The IFA-2::RFP construct was then combined with the LET-805::GFP plasmid in an injection mix using equal DNA proportions of about 50ng/μl for each plasmid. With the typical addition of *rol-6* coinjection marker and carrier DNA, this mix was used in injections of N2 animals to obtain transgenic lines for animals expressing both proteins simultaneously. These animals were observed under both the epifluorescent and confocal laser illumination to investigate a possible protein-protein interaction. The prediction is
that if an interaction with IFA-2 determines where LET-805 is positioned, LET-805 would colocalize to IFA-2 aggregates outside of FOs. However, there was no colocalization of LET-805 at IFA-2 aggregates (Fig. 13). Thus IFA-2 does not appear to determine the positioning of LET-805. One possibility is that LET-805 binds directly to IFA-2, but is constrained at the FOs by interactions with additional proteins not present in the ectopic aggregates.

Fig. 13 – Co-expression of IFA-2::RFP and LET-805::GFP. Photomicrograph of an animal co-expressing IFA-2::RFP and LET-805::GFP. (A) IFA-2::RFP expression results in protein aggregates (arrows) outside of the FOs. (B) The only expression in the green channel for LET-805::GFP, correlating to the IFA-2::RFP aggregates, is bleed-through. Scale bars are 10µm.
Initial Y2H Screen

The observation that the head domain could localize to FOs suggested that it might directly interact with the FO receptors. When first starting the Y2H work I was only interested in looking at a direct protein-protein interaction between IFA-2T with either MUA-3 or LET-805, based on the early GFP expression data that suggested the tail domain was essential to IFA-2 function. The DNA for each potential interactor was cloned into both bait and prey plasmids so the interactions could be investigated in reciprocal fashion and so I could look at each protein possibly interacting with itself.

Transformation of the plasmids into yeast was done in sequential order with the bait constructs transformed first and then those samples were transformed with the prey constructs to obtain the final yeast stocks containing both a bait and prey plasmid along with the pSH18-34 reporter plasmid.

For positive controls, E192 that encodes the second domain of the P6 protein of Cauliflower Mosaic Virus, which is an interaction domain of P6, was used as the bait and E133 encoding the full-length P6 protein, was used as the prey. E192 and E133 were previously found to interact using the Y2H system (S. Leisner, personal communication).

The negative controls were original pEG202 and modified pJG4-5 stocks provided by Dr. Scott Leisner. Yeast already transformed with the E192 positive control bait was provided by Lindy Lutz, along with the E133 plasmid already cloned and ready for transformation.

There were 11 different combinations of transformations performed: the positive control of E192 against E133, the negative control of empty pEG202 against empty pJG4-5, IFA-2T against either itself, LET-805, or MUA-3, MUA-3 against either itself,
LET-805, or IFA-2T, and finally LET-805 against either itself, MUA-3, or IFA-2T. The first attempt at the prey transformation yielded about 300 colonies for all –U/-H/-T plates (suggesting a successful transformation), but only the positive control –U/-H/-T/-L plate had any number of colonies with about 40. Repeating the prey transformation gave similar results, this time with about 100 colonies for the –U/-H/-T/-L plate of the positive control. For a third prey transformation, this time doing an optional resuspension step in preparing yeast cells and increasing the amount of prey plasmids used to transform, we obtained an even greater number of colonies on the positive control –U/-H/-T/-L plate (about 800) and had colonies on all other plates. Though upon further inspection these colonies proved to only be bacterial contamination and not yeast colonies, again suggesting there is no interaction between the proteins.

Before attempting the prey transformation again, I decided to also look at IFA-2H in a possible interaction with itself or the other three proteins already under investigation. After IFA-2H bait and prey plasmids were cloned, I transformed the EGY48 yeast (containing the pSH18-34 reporter) with the IFA-2H::pEG202 plasmid to have a bait-transformed stock to continue work with prey transformations. Even though I believed no constructs were able to auto-activate the Y2H system due to results from the prey transformations so far, I plated the IFA-2H bait transformation onto -U/-H/-L and -U/-H/x-gal plates in addition to regular -U/-H plates to check its ability to auto-activate. As suspected the transformation yielded no colonies on the -U/-H/-L plate (that should require an interaction to compensate for the lack of leucine) and only white colonies on the -U/-H/x-gal plate (that should require an interaction to induce the β-gal activity of the reporter plasmid). So now resuming prey transformations I had 18 total interactions to
look at including the 11 already described, IFA-2H used as a prey against IFA-2T, LET-805, and MUA-3, and IFA-2H as a bait against itself, IFA-2T, LET-805, and MUA-3. The first prey transformation that included the IFA-2H constructs yielded colonies on -U/-H/-T/-L plates other than the positive control, which were assayed for the strength of the interaction. The full set of prey transformations was performed two more times, but again yielded no colonies on -U/-H/-T/-L plates, other than the positive control, that could be assayed for an interaction.

The bait::prey combinations that I was able to quantify a possible interaction by the β-gal assay are plotted in Figure 14. Six to ten different colonies from each plate analyzed were inoculated and assayed to give a larger data set. During the assay the samples were timed as to how long it took them to turn yellow. This timing was kept as consistent as possible in determining the sufficient amount of yellow coloration. Negative control samples timed out at the maximum recording time of 25 minutes while the rest of the samples tested varied within the testing range. For all the interactions, the only ones showing significant difference (Kruskal-Wallis test, \( P < 0.05 \)) from the negative control were the positive control and IFA-2T::IFA-2H.
Fig. 14 – IFA-2 tail weakly interacts with the head domain, but not the matrix receptors MUA-3 or LET-805. All interactions assayed are shown except for the positive control, which had activity levels several logs higher than any interaction and was significantly different from the negative control. Star (★) notes the only interaction determined as significantly different by comparison to the negative control using the Kruskal-Wallis test (P < 0.05). Blue boxes depict the interquartile ranges and whiskers represent the minimum and maximum values for each set of data.

From the β-gal assay, we concluded that there is no direct interaction between LET-805 or MUA-3 with either the head or tail domains of IFA-2. The assay suggests however that the tail domain interacts with the head domain of IFA-2.

Y2H Library Screen

After the β-gal assay, a Y2H library screen was used to discover candidate interactor proteins of IFA-2. With no direct interaction seen between either MUA-3 and
LET-805 receptors with IFA-2, the library screen could identify intermediate proteins that may bridge the gap between membrane receptor and IF.

From the library screen using IFA-2H as bait, we were able to identify 52 yeast clones in our first batch of colonies analyzed from the sterile glycerol stock and 53 samples in the second batch. Including the additional 72 samples identified by using the original glycerol stock, 177 total populations of yeast were analyzed. Since some samples resulted in multiple PCR bands being sequenced, a total of 206 inserts were sequenced and the corresponding worm gene identified by BLAST (Harris et al., 2010). Many of the 206 sequences proved to be duplicates, resulting in 46 unique interactors identified.

Frequency of the interactors ranged from 58 times for CPN-3, to just a single occurrence for most of the proteins identified (Table 2). In general, a true interactor of IFA-2H might be expected to be picked up relatively frequently. While we cannot discount rare occurrences that may represent a true in vivo interaction, the more frequently identified proteins were weighted more heavily as having significance. One of the 46 sequences identified codes for rDNA, while putative proteins were identified for the remaining sequences.
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* Number of bands successfully sequenced

Table 2 – IFA-2H Y2H library screen results. 46 unique interactors were identified in the screen. Brief descriptions are listed. Genes are ordered based on how many times they were identified in the screen by sequencing the corresponding library inserts.

Descriptions for the 45 proteins were gathered from information listed on WormBase (Harris et al., 2010). Using the descriptions, proteins were categorized into six different fields for their presumed primary roles (Fig. 15). Besides assigning relevance of the interactions based on function, we also looked at gene expression profiles using the SPELL database (Hibbs et al., 2007). Perhaps if the interactor in question truly has a role in the FO then we will see it sharing an expression profile with
another member of the complex. The majority of the genes (40/46) had either no connection in expression to FO proteins or were just similar to other genes identified in the screen, while the remaining six genes stood out for their connection to IF genes. No genes had a correlation with the expression profile of *ifa-2*, but six genes correlated with the expression of at least one other IF. These six genes are *tag-60, ucr-2.1, ost-1, unc-44, Y76A2B.5, and pat-12*. For example, *tag-60* shares expression profiles with the intermediate filaments *ifb-1, ifb-2, and ifd-1*. An orthologue of *tag-60* in Drosophila is essential for epidermal integrity (Hughes et al., 2010).

**Fig. 15 – Functional grouping of IFA-2H interactors.** The 45 proteins identified from the library screen using the head domain of IFA-2 were grouped into 6 categories for presumed primary roles, based on sequence identity and phenotypes reported in WormBase.
Our final criteria in determining relevance was based on reported tissue expression. Most of the data came from anatomical expression patterns detailed in WormBase. Common sense would dictate that if an interactor of the Y2H library screen is vital to the function of IFA-2, then it will be expressed in the same tissues. Our main focus has been on the function of IFA-2 in FOs of the hypodermis, but IFA-2 is also found in the vulva, pharynx, and as reported by some, nervous tissue (Karabinos et al., 2002). Comparisons to IFA-2 tissue expression could only be made for 21 of the 45 proteins identified, as the rest did not have reported expression patterns (Fig. 16). Of these 21 proteins, only five were reported to have hypodermal expression, on the other hand, only three did not share any type of tissue expression with IFA-2, which also includes pharyngeal, vulval, and nervous tissues. The biggest issue with this data is that it is incomplete. If tissue expression is not reported it may not have been looked for in that tissue, or at the appropriate stages.
### Table: Expression Patterns

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**Fig. 16 – Expression patterns of IFA-2H Y2H interactors.** Comparison of available expression data from WormBase for 21 interactors compared to IFA-2 tissue expression. Dark blue boxes correspond to relatively high levels of expression, while light blue corresponds to relatively low levels of expression, as reported by investigators on WormBase. Remaining blue boxes represent expression that has not been characterized. Nervous expression for IFA-2 has not been confirmed by our lab.
One previously identified FO protein, PAT-12, was identified in the IFA-2H library screen. PAT-12 has already been established as a member of the FO. Being able to pull out a member of the FO in this library screen would suggest that we are capable of identifying proteins that interact with the head domain of IFA-2.

The library screen identified 45 interactors of IFA-2H that need further investigation to establish a possible role within the FO. Though some proteins appear more relevant to the FO than others, such as those labeled as structural proteins, most proteins in the screen match at least one of the criteria examined, and could be worth further investigation.

The library screen was also performed using IFA-2T as bait, but due to the inefficient transformation, only 14 sequences were identified. Unlike the IFA-2H library screen, all 14 sequences were unique and their corresponding genes can be seen in Table 3. In addition, unlike the previous library screen, no stock was prepared from the library transformation with IFA-2T. So the results reflect all possible yeast colonies that could be analyzed from that transformation. These results were not further analyzed using the criteria described earlier for the IFA-2H interactors. Most of the 14 interactors identified were unlikely to have a role in cell adhesion. Though one protein stood out in the screen, which was the hypothetical protein K04G7.1. This protein was also identified in the library screen using IFA-2H as the bait, suggesting K04G7.1 has a role within the FO, which may include either the head or tail domain of IFA-2.
Table 3 – IFA-2T Y2H library interactors. WormBase sequence names and descriptions for interactors found in library screen using the tail domain of IFA-2. Each interactor of the screen was identified a single time.

K04G7.1 Expression

One of the proteins identified from the IFA-2H Y2H library screen was K04G7.1. This is a hypothetical protein which has been described in little detail. While no information suggested a role for K04G7.1 within the FO, it had a high frequency of recovery at being identified eight times in the IFA-2H screen, and had the distinction of being the only protein to be identified for both IFA-2H and IFA-2T library screens. Because of the possibility of working with either the head or tail domain and because so little was known about the protein, I decided to further investigate K04G7.1.

So I tagged the protein with GFP to look for an expression pattern. In order to determine whether this interaction with IFA-2 is likely to be taking place in vivo, I
wished to determine if it was expressed in the same tissues as IFA-2. Transgenic lines for k04g7.1::gfp were established through typical means of microinjection and animals of all stages were observed under the compound scope, to define an expression pattern. Lines originating from individual F1 animals where observed separately to look for any variation in protein expression, but all lines proved to have similar patterns.

Expression of the protein was limited to pharyngeal tissue and was not observed anywhere else in the animal (Fig. 17). The typical expression pattern is a diffuse staining throughout the pharynx from procorpus to the terminal bulb. This pattern is seen in all stages from L1-L4 and adults. Along with the typical expression pattern, 6% (3/48) animals show higher expression levels in only certain parts of the pharynx. In the case of a L4 animal with bright expression throughout the corpus section of the pharynx or a L2 with bright expression in only the procorpus segment of the corpus, this may occur during molt stages, as both animals appeared to be undergoing a molt when observed under the transmitted light of the compound scope. Though in the case of an adult with high isthmus expression, this irregular bright expression localized to segments of the pharynx does not always occur during molts. Expression was not observed for the few embryos examined.

While K04G7.1 did not localize to FOs, it was found expressed throughout pharyngeal tissue, another tissue common to IFA-2 and other IFs of the FO. Suggesting a possible involvement with IF function in this tissue.
Fig. 17 – Representative photomicrographs of animals expressing K04G7.1::GFP. Expression is typically found throughout pharynx from procorpus to terminal bulb (A). Irregular bright expression in segments of pharynx is found in some animals. (B) L2 animal with bright procorpus expression, (C) L4 with expression throughout corpus, and (C) adult with expression localized to isthmus (D). Scale bars are 20µm.
4. Discussion

While *in vitro* work has made the mechanics of IF assembly clear, little is known about the regulation of this assembly and how IFs are incorporated into FOs. This work has established the rod domain as being essential to IF dimerization and subsequent filament maturation, however the roles of the other domains in IFs are less well-defined (Hatzfeld and Weber, 1990). In addition, because IFs are differentially expressed, their head and tail domains are likely to have unique functions, making it difficult to ascertain roles for the domains. I was interested in understanding the role of the head and tail domains in interactions with other FO proteins and within the adhesion complex. This study focused on the intermediate filament protein IFA-2 although other IF proteins also are found within the FO. In the FOs, IFA-2 is likely to exist as a heterodimer with IFB-1, as are IFA-1 and IFA-3. It is possible while the rod is essential to dimerization, the other domains may serve some function in IF-IF interactions and perhaps determine which IFs will specifically dimerize. To investigate the roles of the head and tail domain, two complementary approaches were taken. First, constructs expressing only specific portions of IFA-2 domains were generated and expressed in animals as translational reporters tagged with GFP, to observe the effects of these constructs on IF maturation and assembly into FOs, and on IFA-2 function. Expression of these constructs would also allow me to observe any deleterious effects they may have on the animal as a whole,
when over-expressed. The other approach was to look directly for protein-protein interactions with the IFA-2 head and tail domains, initially using yeast two-hybrid to identify candidates, and then testing the candidates \textit{in vivo}.

The IFA-2 head can localize to FOs whereas the tail cannot

Constructs expressing the full-length IFA-2, IFA-2 deleted for the head region, or IFA-2 deleted for various portions of the tail domain were previously generated and examined (Hapiak et al 2003; Williams, 2008; Baucher, 2010). The full-length \textit{ifa-2::gfp} transgene is functionally identical to wild-type IFA-2, which is important as it suggests that GFP-tagged constructs will behave similarly to the native IFA-2. The three important characteristics noted was the ability of IFA-2::GFP to localize to the FO, of the construct to rescue the \textit{nc16} null allele of \textit{ifa-2}, and that there was no obvious novel or dominant-negative phenotype associated with expression of the construct (Hapiak et al., 2003). Also of note is that the IFA-2::GFP expression pattern is identical to the staining pattern seen with the MH4 antibody which recognizes several epidermally expressed IFs, including IFA-2. The IFA-2\textDelta H::GFP variant deleted for the head domain is functionally indistinguishable from the full-length construct, when expressed \textit{in vivo}. This variant is also always localized to FOs, can rescue for the null, and has no phenotype (Williams, 2008).

On the other hand IFA-2\textDelta T::GFP, which is the IFA-2 variant with the tail domain removed, is unable to rescue the null and results in a Mua phenotype in rare cases (Baucher, 2010). This variant localizes to FOs, but its expression there is drastically lowered relative to intensity levels of the full-length and \textDelta H constructs. One of the
problems with microinjection is that the resulting expression can be variable since the number of copies of the transgene being uptaken can fluctuate. This could explain why expression of the ΔT construct is less than the full-length and ΔH constructs. This possibility is attenuated by the observation that all animals of a line display consistent intensity levels. The fact that IFA-2ΔT cannot rescue the null and occasionally leads to a dominant-negative Mua phenotype when expressed at high levels in an ifα-2<sup>+</sup> background, suggests that the tail domain of IFA-2 has an essential role. The construct is still able to localize to FOs, but perhaps inefficiently without the tail domain and is unable to function at wild-type levels.

In contrast, the head domain appears dispensable as the IFA-2ΔH variant performed identically to the full-length protein. Since it is unlikely that the head has no function, either its function is redundant with some other IFA-2 domain or other protein, or its function does not pertain to FO function. In vertebrate neuronal IFs, the head domain is able to specify the heterodimeric partner of assembly, but by what interaction has yet to be seen (Ching and Liem, 1999). This interaction may be redundant, as my transgenic expression of IFA-2 variants suggests, and because of the fact that the head domain of a vertebrate lamin is able to substitute for the head of a neurofilament, in IF assembly. Though substitution by both head and tail domains of the lamin resulted in defective IF assembly, again corroborating my work suggesting the function of the tail domain is unique to IFA-2 (Gu et al., 2004).

In this thesis I showed that, the head is able to localize to FOs on its own, suggesting a direct interaction with a protein of the complex. Conversely, I showed that the tail is unable to localize to FOs on its own. While not eliminating the possibility that
it can interact with FO proteins, it suggests the tail depends on the rod domain for these interactions to occur.

Based on keratinocyte *in vitro* cell culture studies, it has been shown that the rod domain is the essential portion of IFs that allows for dimerization and maturation of filaments (Strelkov et al., 2003). Likewise, I have shown in this thesis that the rod domain alone can mediate incorporation of IFA-2 into the FOs. The IFA-2R::GFP variant was able to localize to FOs, although clearly with less efficiency than wild-type. This suggests that while the rod domain allows IFA-2 to localize properly, the missing head and tail domains are contribute to IFA-2 localization, possibly by assisting with dimerization or by interactions with accessory proteins within the FO. Predicted phosphorylation sites on IFs are clustered at the head and tail domains, suggesting a role for regulation of IF incorporation by posttranslational modification (Omary et al., 2006). This dysregulation may be apparent in vertebrate IFs mutated in the tail region, resulting in abnormally thick filaments (Ching and Liem, 1998). Phosphorylation of the head domain is able to inhibit phosphorylation of the tail, though if this is due to an interaction or through a secondary messenger, is not clearly understood (Zheng et al., 2003). Head and tail domains also appear to function antagonistically in IF assembly where the head promotes assembly, while the tail terminates the assembly, at precisely 10nm filaments (Heins et al., 1993). It is also possible that IFA-2R::GFP could be associating with IFs already assembled within the complex instead of taking part in the dimerization and maturation of filaments, accounting for its partial incorporation at FOs. If the head or tail domain is involved in targeting the proto-filaments in the FOs, we expect to see these
domains localize to FOs without the rod domain. This is apparently true of the head domain.

IFA-2H was able to localize to FOs, but again in only a subset of animals observed. Along with the reduced frequency of localization, IFA-2H was found to properly localize only in larval stages. In adult animals, expression was restricted to hypodermal ridges. The head domain may interact with a protein within the FO to allow for its localization, but this interaction may either not be strong enough to keep the protein there during adult stages or it is simply displaced from the FO by native IFA-2. Endogenous IFA-2 likely has stronger interactions with the FO because of the rod and tail domains being present and it may act synergistically with its dimerization partner IFB-1 to associate with accessory proteins and strengthen the IF network. At the adult stage these stronger interactions finally override any interaction IFA-2H is able to make within the FO. Alternatively, IFA-2H::GFP may not be found at FOs in adults because these animals that show this localization are Mua and do not make it to adulthood.

Overexpression of IFA-2H appears to result in a Mua phenotype. This phenotype was correlated with bright expression of IFA-2H localized to FOs. In its normal context, it is likely the head domain strengthens the IF network by anchoring the filaments to receptors at the apical and basal hemidesmosomes or by interacting with opposing domains of the dimerization partner.

One model that explains the earlier observation that the head domain appears dispensable for IFA-2 function is that head domains of different IFs may serve a redundant role where one can compensate for loss of the other, since IFs are found as heterodimers. This explains why IFA-2ΔH is indistinguishable from wild-type IFA-2,
because the head domain of IFB-1, the dimerization partner of IFA-2, is able to compensate. Therefore the head domain is sufficient for interaction with the FO, but is not essential. Without the presumed dimerization qualities attributed to the rod domain, the head domain localizes to FOs because of a direct interaction with another protein in the FO. This interaction may be identical to ones involving head domains of other IFs.

In contrast to the case where the head domain appears to be redundant, but clearly can bind to the FOs, surprisingly the tail domain, which is essential for IFA-2 function, cannot by itself bind to the FOs. IFA-2T::GFP did not localize to FOs at any stage of development, in any of the observed animals. Expression was still found at hypodermal ridges, similar to IFA-2H at the adult stage, showing that this transgene is expressed in the correct tissues. Our previous model suggested that since the tail domain appeared essential to IFA-2 function, as it was necessary for rescue of the null allele and wild-type levels of expression, that it may have a unique function specific to IFA-2, distinct from at least some of the functions of the other epidermally expressed IF protein’s tail domains. Based on my IFA-2T::GFP expression we cannot conclude that the tail domain alone is able to bind the FO, in vivo. The construct was never found at FOs, so whatever its function may be, it apparently requires the rod domain to localize to the FOs. Another scenario is that the tail domain interacts with other IFs to strengthen the dimerization, but again this may be dependent on the rod domain. This would explain why loss of the tail domain of IFA-2 could not be compensated, because the tail domains on opposing IFs interact to strengthen the mature filaments. It would be interesting to see if tail domains of other IFs have similar attributes, as I predict they are unique to the IF. Perhaps other tail domains can localize to FOs with interactions independent from the rod domain or
maybe they also serve to strengthen IF dimerization by binding to opposing tail domains. Further analysis such as our Y2H screen would serve to shed light on what role this tail domain serves. Based on this and our previous expression data, the one thing that is clear is that both the tail and head domains do serve a purpose. The rod domain is enough for some degree of dimerization and localization of IFs to FOs. While the head domain has some interaction within the FO, the tail domain is essential for wild-type function of IFA-2 that is dependent on the rest of the protein. The tail domain may function in regulating the incorporation of IFs into the FOs. SUMOylation of IFB-1 serves to maintain a cytoplasmic pool of IFs for exchange with filaments incorporated at FOs, and this SUMOylation takes place at the tail domain (Kaminski et al., 2009).

**IFA-2 does not directly position the membrane receptor LET-805**

LET-805 is a well-documented member of the FO and as a membrane receptor serves as a probable interactor with IFs (Hresko et al., 1999). While it has been established that LET-805 expression results in the circumferential striping pattern typical of FOs, any possible interactions have not been validated for the protein. Because LET-805 expression colocalizes with that of MH4 antibody stained IFs, it is likely the receptor and IFs interact in order to establish FO positioning. My LET-805::GFP fusion protein resulted in a typical FO pattern, suggesting that LET-805 does not require its extracellular domain to be positioned at the FOs. This is consistent with models in which the IFs or other FO associated proteins can directly bind and position the matrix receptors (Hong et al., 2004). However, LET-805::GFP did not colocalize with ectopic epidermal accumulations of IFA-2::RFP, suggesting that IFA-2 does not directly position the
membrane receptor LET-805. Transgenic lines co-expressing both LET-805::GFP and IFA-2::RFP were able to generate the ectopic IFA-2 aggregates. Under epifluorescence by the compound scope all cases of green fluorescence that coincided with the red protein aggregates of IFA-2 proved to be bleed-through from the red channel. We also looked at some animals under confocal microscopy, but were left with the same results of no LET-805 localizing to the aggregates.

It has previously been established that expression of IFs can lead to protein aggregates within the tissue that are not a part of the typical FO striping (Woo et al., 2004). These accumulations are likely a result of interactions with the normal IFA-2 binding proteins being saturated. All exogenous protein incorporates into the adhesion structures and the excess ectopically accumulates. Our logic was that if LET-805 is able to interact with IFA-2, then it will be found colocalized to these protein aggregates.

This negative result does not conclude there is no interaction between LET-805 and IFA-2 as there are many possible explanations. One drawback of this experiment is that the accessibility or structure of IFA-2 in the ectopic aggregates may differ from IFA-2 seen in the FOs. Other possibilities are that fluorescent tags could be preventing the interaction, but this is unlikely due to the fact that the proteins are able to colocalize at FOs. Aggregates of IFA-2 may not be in a native conformation that is normally accessible to LET-805 in the case at FOs. Although there is a surplus of IFA-2 leading to aggregates, but perhaps all of the exogenous LET-805 is still being localized to FOs. The interaction may be indirect and requires another member of the FO that is anchored at the FO and unable to be recruited to the aggregates.
The IFA-2 tail domain interacts with the head domain

Using a genetic approach I had shown that the IFA-2 protein is likely to bind to proteins of the FO. In addition, although I did not observe localization of the IFA-2 tail with the FOs in the absence of the rod domain, this does not rule out the possibility the tail can bind to FO proteins when brought into the filament by the rod. Finally, despite the observation that IFA-2 and LET-805 did not appear to interact with each other in an ectopic location it is still possible that LET-805 could bind IFA-2 when both are in the context of the FO. Therefore I used the Y2H system as another method to evaluate whether interactions between the cytoplasmic domains of two major FO receptors and the head and tail domains of IFA-2 were possible. It is also clear that the intermediate filaments of the FO must ultimately be physically linked to the FO matrix receptors either directly or via intermediary proteins. If IFA-2 interacts with the basal lamina through the basal membrane receptor LET-805, then we would also expect an interaction to take place with an apical receptor. The apical portion of the FO has two membrane receptors, MUA-3 and MUP-4, unlike the single receptor found on the basal side (Bercher et al., 2001; Hong et al., 2001). MUA-3 was examined for a possible interaction with IFA-2, as both are required post-embryonically, while MUP-4 is essential during embryogenesis. In addition, by examining interactions using the individual domains of IFA-2, we could distinguish whether the two receptors bound to different portions of IFA-2. Because I was focusing on discovering roles for the head and tail domains, interactions between the rod domain of IFA-2 and other proteins were not examined in this experiment.

Typically the biggest issue in the Y2H system as a means for identifying protein-protein interactions is that it is always prone to false positives (Serebriiskii et al., 2000).
Two proteins may interact in Y2H, though in vivo these interactions might be excluded due to the timing of their expression, their localization, or the tissues that they are expressed in, making any possible interaction irrelevant. False negatives may occur because the fusion of the proteins to DNA-binding and activation domains could alter their conformation, posttranslational modifications that are necessary for an interaction may be absent, and the localization of cytoplasmic proteins to the nucleus of the yeast may be prevented. False negatives may have been a part of this experiment, as we had difficulty in getting interactions to take place. Most interactions detected were not above background, and those that were showed weak interactions. For one set of prey transformations I was able to get growth on –L plates for seven of the interactions tested, besides the positive control. There was no sign of auto-activation, at least with IFA-2H, arguing against the idea of false positives.

β-gal values were analyzed using the Kruskal-Wallis non-parametric test. Some samples analyzed within the set tested for a specific interaction were completely out of line (possible outliers) with the majority of the set. The Kruskal-Wallis test ranks samples, allowing us to use all of the data, but minimizes the effects of potential outliers.

The positive control was several logs of scale higher than any other interaction assayed (data not shown). It was confirmed that the activity of the positive control was significantly different from the negative control (P value < 0.05). This control consisted of two plant viral proteins previously confirmed in Y2H to interact (S. Leisner, personal communication), making it difficult to compare to interactions taking place in a different system, in a different organism. Quite possibly why the strength of interactions of the C. elegans proteins were much lower compared to the positive control is because they are
naturally weaker interactions *in vivo*. In the FO it would be expected that many of these interactions would be taking place with not only many IFA-2 filaments, but also the other filaments present. These interactions may be weak individually, but as a collective whole they serve to strengthen the adhesion complex.

The interaction between the tail and head domains of IFA-2 was the only other interaction, besides the positive control, that was significantly different from the negative control interaction (P value < 0.05). Because the mature filaments in the FO consist of many IFs bundled together, it is plausible that these globular head and tail domains of the IFs interact to serve as another anchor in that complex to hold the filaments together and strengthen them against the stress generated by the muscle. Cases do exist where the head and tail domains possess a coordinated function. In the vertebrate IF, desmin, the tail interacts with the head, acting as a cap, in order to prevent cleavage at the amino terminus of the filament (Mavroidis et al., 2008). So there exists multiple possibilities to reason for an interaction between the head and tail domains of IFA-2.

It appears neither LET-805 nor MUA-3 interact with either IFA-2H or IFA-2T, at least directly. This data corresponds with the previous expression data that LET-805 did not colocalize with ectopic IFA-2. MUA-3 had more possible interactions assayed, though all interactions were not significant, according to the statistical analysis. It could be the MUA-3 protein expressed in the Y2H system is just intrinsically susceptible to binding with other proteins. Any *in vivo* interactions for the membrane receptors and IFs likely require an intermediary.

A next step in confirming an interaction between the head and tail domains, seen in the Y2H experiment, could be to express these proteins in *E. coli* and attempt to
immunoprecipitate the proteins as a complex. In addition to testing for an interaction between IF domains, accessory proteins within the FO such as VAB-10 could be included, to test the possibility of an intermediate bridging the interaction between receptor and IF.

A Y2H cDNA library screen has identified 59 candidate IFA-2 interacting proteins

I used a Y2H cDNA library screen using the head and tail IFA-2 domains as the bait and a *C. elegans* cDNA library as a prey, to look for any novel protein interactions with IFA-2, and potentially replicate FO/IF interactions reported in the literature. The screen also had the potential to detect an interaction with known FO proteins that have yet to be shown to directly interact with the IFs. Based on the GFP reporter studies showing that the isolated head domain but not the isolated tail domain could localize to FO, I primarily focused on using the IFA-2H as the bait when screening the library. This screen identified 239 total positive hits using the head domain as bait, that represented 45 different proteins, and 10 hits representing 10 proteins, using the tail as bait.

Looking at the actual results from the library screens my attention has been focused on the results using IFA-2H as the bait versus IFA-2T, which were much less fruitful. For the IFA-2H screen 239 unique yeast populations were analyzed, resulting in 46 unique sequences. Meanwhile for IFA-2T I was only able to look at 28 samples, from which we obtained 14 sequences. Thus I was able to get a more complete picture of interactions taking place with the head domain of IFA-2, with many multiple hits in the same proteins. While all the tail hits were unique, none of the candidate proteins that were identified as potential tail domain partners are compelling candidates for a genuine
in vivo interaction with IFA-2. Many of the hits were genes that code for broadly expressed enzymes or proteins involved in protein synthesis. These include GDH-1, a glutamate dehydrogenase, and RPS-5, a ribosomal subunit. It is difficult to rationalize a specific role for these proteins in maintaining tissue integrity. Examples from the library screen with IFA-2T that would be discounted as having relevant interactions with IFA-2 include the ribosomal proteins RPS-5, RPS-16, and elongation factor EFT-2. While these proteins are likely within the cytoplasm of the hypodermis along with IFs, there is no evidence to suggest a role in tissue integrity. Though it is possible these ribosomal proteins interact with IFs in preparation of synthesizing new filaments, to strengthen the FO. Other proteins like GDH-1, the ATP synthase ATP-2, and the cytochrome c oxidase subunit MTCE.31 are likely restricted to a compartment separate than that of IFs, in this case the mitochondria, again arguing against a relevant interaction for the proteins in vivo.

The only two proteins identified in the IFA-2T screen that are plausible candidates to interact with IFA-2 in vivo are the hypothetical proteins C09G9.2 and K04G7.1. The WD40 domain of C09G9.2, which is the domain present in the positive Y2H prey clone, is reported to have function in cytoskeleton assembly (Collier et al., 2005). Little else is known of C09G9.2. However, in a single RNAi experiment, reduced expression of the protein led to larval arrest and lethality. These phenotypes are common to ifa-2 null animals with a Mua phenotype, but are too broad to narrow down C09G9.2 as having a function specific to tissue integrity (Kamath et al., 2003). K04G7.1 was also identified as interacting with the head domain and will be discussed later.
The IFA-2H library screen resulted in many more hits, with 45 proteins that fell into a variety of functional groups. Plausible candidates were identified based on multiple criteria. The most important criteria were functional grouping, expression patterns (where known), hit frequency, phenotype (when known), and known functions of homologous proteins in other species. 46 sequences were initially identified, but 1 proved to be a rDNA tandem repeat. Proteins grouped into a structural field were most relevant to our work in adhesion and tissue integrity, though not all of the other proteins can be dismissed, especially when the FO may serve other roles including signaling.

When taking all of the criteria into consideration, three proteins stood out. The calponin orthologue, CPN-3, was remarkable, simply for the fact that it was identified 58 times in the screen, by far more than any other protein. This frequency suggests that CPN-3 strongly interacts with the head domain to serve some purpose. Although not found to be expressed in the hypodermis, CPN-3 is present in the vulval and nervous tissues in common with IFA-2. Calponins are reported to bind actin (Ferjani et al., 2006), another cytoskeleton filament, so perhaps CPN-3 is also capable of binding IFs, as suggested by our library screen. CPN-3 has already been attributed to possess ATPase activity, which suggests a possible role in muscle contraction, not cell adhesion (Lin et al., 1993).

Another intriguing interactor identified was FLN-1. This filamin orthologue is expressed in all tissues, had multiple hits in the screen, and has reported roles in cytoskeleton remodeling. The protein has been established to function in cross-linking actin filaments (DeMaso et al., 2011).
Finally, the most interesting result may be the identification of TAG-60. The orthologue of this protein serves to maintain epithelial integrity in Drosophila, comparable to the role of FOs in hypodermal integrity of C. elegans (Hughes et al., 2010). The protein also had a high frequency in the screen at 18 hits and correlates to gene expression of \textit{ifb-1, ifb-2, and ifd-1} (Hibbs et al., 2007). However, the only tissue expression correlating to IFA-2 expression is the pharyngeal expression, so it is unlikely to be found at FOs. Ultimately, arguments could be made for a connection to IFA-2 for most of the interactors identified. The Y2H system can only be used as a tool for exploring the possibility of protein-protein interactions. Subsequent work is necessary for following up the data and exploring the mechanism of interactions and what these interactions could mean to the FO as a whole.

Variation may have been limited in the Y2H library results with IFA-2H as the bait, since the first 156 yeast clones analyzed came from a glycerol stock made from just two prey transformation plates. For the library screen results with IFA-2T, all interactors identified may possibly have been false positives, as the control plates and small number of colonies suggest that the prey transformation was unsuccessful.

As common with Y2H, there were some false positives with only a background false positive rate of 7% of samples restreaked onto x-gal plates and only 4% of samples failing to be PCRed. The most likely reason why the samples had blue growth to begin with is because of the bait’s transcriptional activity (Serebriiskii et al., 2000). These samples somehow managed to activate transcription for downfield reporters, even though no interaction between the bait plasmid containing the DNA-binding domain, and the prey plasmid consisting of the activation domain, was made. The fact that baits were not
able to auto-activate in the control tests is in line with the low occurrence rate. Upon the second x-gal streaking whatever activation was possible the first time is now lost and false positives are revealed.

In 19% (37/190) of the clones analyzed, multiple library inserts were present, as indicated by the presence of multiple PCR bands. Samples are restreaked multiple times after the transformation to segregate multiple copies of inserts, but perhaps some samples just had too many inserts to be segregated or the restreaking was ineffective at removing multiple copies. Another explanation for the multiple PCR bands is that they are truncations of the full-length sequence, substantiated by the fact that multiple bands within some samples were found to code for the same gene.

It is difficult to assess the relevance of these multiple inserts. 6 of the 19 proteins present in yeast samples with multiple library inserts do not match any of the criteria used to analyze all samples in the screen. These include the ribosomal protein RPL-2, nuclear hormone receptor NHR-202, mitochondrial protein FAT-1, transport protein TTR-25, signal recognition particle protein F21D5.7, and a hypothetical protein Y71H2AM.5. Though other proteins found in samples with multiple inserts include K04G7.1, CPN-3, and OST-1, which were highly regarded in the screen. In some cases, both inserts were highly-regarded proteins, some clones had a mix of one protein of interest and one that did not appear relevant to cell adhesion, and some clones had inserts that both corresponded to irrelevant proteins. Therefore, no significance was assigned to whether PCR bands were amplified individually or were PCRed in conjunction with multiple samples.
K04G7.1 is expressed in pharyngeal tissue

The hypothetical protein K04G7.1 was the only protein identified in both the head and tail domain library screens. It was fairly common with eight hits in the IFA-2H screen. While this data supports a role in interacting with the head or tail domain or both, there is not much else known about the protein. There is no database information on expression and BLAST searches conclude there is no homology to other proteins, but analysis of domain architecture by SMART determines there is a single transmembrane segment from amino acids 388 to 513 (Schultz et al., 1998). RNAi information in the databases does not provide a revealing phenotype either. Thus K04G7.1 provides a good model for handling other candidates in this class.

Fluorescence of the translational GFP-reporter was restricted to the pharynx of all stages and could not be observed in any other tissues. The typical pattern was of a diffuse staining throughout the pharynx from procorpus to terminal bulb. Elevated expression was found in just the procorpus, corpus, and isthmus regions in three separate animals, which may correlate with a timing event, as two of the animals appeared to be undergoing a molt. The fact that K04G7.1 does not co-localize with IFs directly at the basal-apical axis of the marginal cells of the pharynx, argues against a role for K04G7.1 in the FO-like structures of the pharynx (Woo et al., 2004). Expression throughout the pharynx may suggest a role for the protein in the feeding process. K04G7.1 could code for a digestive enzyme in breaking down food, or it may code for a signaling molecule that coordinates the contractions of the pharynx during pumping. These molecules are likely to be found in other organisms, so the fact that K04G7.1 shows no homology to
other proteins or known enzymatic domains, would argue against these possible roles for K04G7.1.

Even though it is not at the FO-like structures in the pharynx, this uncharacterized protein may serve as a molecule capable of regulating IF incorporation, such as sequestering cytoplasmic pools of IFs away from the FO-like plaques in the marginal cells, in a manner similar to the SUMOylation of IFs (Kaminski et al., 2009). Additional experiments are needed to confirm a possible interaction among K04G7.1 and IFA-2, or any other IF. For example one test would be to isolate pharyngies and immunoprecipitate K04G7.1 using the GFP tag and immunoblot with MH4, to detect an in vivo interaction with IFs. Another experiment would be to use RNAi against K04G7.1 in order to look for a disruption in the IF organization.
References


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