PAT-12, AN ATYPICAL PAT PROTEIN INVOLVED IN FIBROUS ORGANELLES IN C. ELEGANS

BY

JONATHAN RHINE

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cell and Developmental Biology in the Graduate College of the University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Associate Professor Michel Bellini, Chair
Associate Professor Benjamin Williams, Director of Research
Associate Professor Phillip Newmark
Associate Professor Charles Cox
Assistant Professor Rachel Smith-Bolton
ABSTRACT

Forces generated by body wall muscles of Caenorhabditis elegans must be transmitted across the worm’s thin hypodermis or “skin” to the cuticle during locomotion. Fibrous organelles (FOs), structures analogous to vertebrate hemidesmosomes, link intermediate filaments to both the apical and basal membrane of the hypodermal cells and form key elements in this force transduction pathway. Here we molecularly isolate the pat-12 gene and show that it encodes a component of the fibrous organelles. The pat-12 gene was identified through the pat-12(st430) mutation, which causes the embryonic body wall muscle cells to rip free from the body wall soon after they first begin to contract during mid-embryogenesis. The pat-12 gene, which corresponds to the complex proposed gene T17H7.4, is predicted to encode 12 different isoforms. The st430 mutation alters the conserved splice acceptor site of exon 12. The PATc12 protein localizes to hypodermal cells in wild-type embryos, but is absent in st430 homozygotes. Here, we show that two of the four isoforms that normally include exon 12 localize to circumferential hypodermal bands characteristic of fibrous organelle components. Furthermore, we propose that PAT-12 forms part of a linkage between the fibrous organelles and the circumferential actin bands (CFB).
To Amy, without whose support this would not have been possible
I would like to thank Dr. Ben Williams for intellectual support and guidance throughout my graduate career. Thanks to former lab mates, Dr. Craig MacKinnon, Dr. Xinyi Lin, and Dr. Qian Liu for input and help with experiments. Thanks to my committee for support and encouragement during my unorthodox path to project completion.
TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION .....................................................................................1
  Hemidesmosomes and the Dermal-Epidermal Junction Zone ......................... 1
  Epidermolysis Bullosa ....................................................................................... 2
  Basal Epidermal Layer ....................................................................................... 3
  Epidermolysis Bullosa Simplex (EBS) ................................................................. 4
  Kindler Syndrome ............................................................................................. 6
  Basement Membrane Layer .............................................................................. 7
  Junctional Epidermolysis Bullosa (JEB) ............................................................. 7
  Dermal Connective Tissue Layer ...................................................................... 8
  Dystrophic Epidermolysis Bullosa (DEB) .......................................................... 9
  Studying Adhesion Complexes in C. elegans ................................................... 10
  The Pat Phenotype and Muscles ...................................................................... 12
  Figures .............................................................................................................. 15

CHAPTER 2: IDENTIFICATION OF T17h7.4 AS pat-12 .........................................18
  Introduction and Rationale .............................................................................. 18
  Materials and Methods ................................................................................... 19
  Results ............................................................................................................. 21
  Discussion ...................................................................................................... 24
  Figures .......................................................................................................... 27

CHAPTER 3: PAT-12 PROTEIN ............................................................................30
  Introduction and Rationale .............................................................................. 30
  Materials and Methods ................................................................................... 31
  Results ............................................................................................................. 36
  Discussion ...................................................................................................... 40
  Figures .......................................................................................................... 42

CHAPTER 4: DISCUSSION ....................................................................................47
  Summary .......................................................................................................... 47
  Summary of PAT-12 Paper by Hetherington et al ........................................... 48
  Predicted Interactions and Model of PAT-12 Function ..................................... 50
  Comparisons with Vertebrate Hemidesmosomes .......................................... 52
  Questions for Future Study ............................................................................ 53
  Figures .......................................................................................................... 56

REFERENCES ....................................................................................................58
CHAPTER 1: INTRODUCTION

Hemidesmosomes and the Dermal-Epidermal Junction Zone

To maintain tissue integrity in the presence of forces, both internally and externally generated, a cell employs several types of physical linkages with neighboring cells and the underlying lattice of the basement membrane. These linkages, such as the vertebrate hemidesmosome, have been extensively characterized as to their protein components and interactions. What we hope to understand is how these complexes are formed and correctly localized during times of transition, such as development and wound healing.

Vertebrate hemidesmosomes (HDs) are 0.1 – 0.5 micrometer structures at the basal membrane of epidermal cells as well as other cells which, when examined by electron microscopy, appears as two electron dense regions (Borradori and Sonnenberg, 1996). Along with the epidermis, hemidesmosomes are found in the cornea, amnion, and parts of the GI and respiratory tracts. (Borradori and Sonnenberg, 1996). Functionally, they link the intermediate filaments on the intracellular side to the extracellular matrix in the dermal-epidermal junction zone (DEJZ) [Figure 1.1]. This region consists of the lamina densa (electron dense region) and lamina lucida (electron sparse region) of the basement membrane as well as the underlying connective tissue regions.

To a first approximation, the connections from the epidermal cells to the dermal connective tissue are as follows: the intermediate filaments of the epidermal cytoskeleton connect to the hemidesmosome. This in turn attaches to the anchoring filaments, which span the lamina lucida of the extracellular matrix. The anchoring fibrils link to these filaments in the lamina densa. The fibrils themselves extend either to anchoring plaques
in the connective tissue or loop back to the lamina lucida again. Collagen fibrils can be caught in these fibrils loops, thus conferring even more strength to the connections (Bruckner-Tuderman et al. 1999).

**Epidermolysis Bullosa**

When these complexes are aberrant or completely missing, blistering occurs at the layer of the erroneous protein in response to applied stress. This leads to a group of disorders collectively known as epidermolysis bullosa (EB). First described in 1886 by Heinrich Koebner, EB affects approximately 1 in 50,000 individuals in the United States (Fine et al, 2008, Prockop and Malwina, 2008). Molecularly, EB is due to mutations in at least 10 proteins, which are members of the hemidesmosomes or their attachments within the basal lamina (Sawamura at al. 2010, Nagy and McGrath 2010, Fine et al. 2008). The prevalence of blisters and their localization varies highly depending on both the aberrant protein and the causal mutation. Phenotypic variation occurs even in individuals with similar mutations leading to the thought that the ultimate disease state can be affected through modification by other related proteins.

In addition to epidermal blistering, patients with EB develop extra-dermal manifestations of the disease as well. Most striking among these is “mitten deformities,” which refer to progressive webbing of the fingers (pseudosyndactyly) (Fine and Mellerio 2009). Surgical correction of these hand deformities through de-gloving procedures is possible and has good results, but must be repeated every 2 years, on average (Fine and Mellerio 2009). Muscular dystrophies, osteoporosis, dilated cardiomyopathy, enamel hypoplasia, delayed puberty, anemia, frequent infections, and squamous cell carcinoma
are other possible systemic manifestations in these patients (Fine and Melleria 2009).
Activities of daily living can be limited due to pain in showering, dressing, etc.

EB was classified into major types based on the blistering pattern and the aberrant protein at the Third International Consensus Meeting on Diagnosis and Classification of EB. These four classifications are epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), dystrophic epidermolysis bullosa (DEB), and Kindler Syndrome (mixed epidermolysis bullosa) (Fine et al. 2008). Additionally, an autoimmune blistering disorder termed epidermolysis bullosa acquisita (EBA) has been described (Lehman et al. 2009).

Basal Epidermal Layer

The basal epidermal layer is the site of the stem cells that give rise to the overlying layers of stratified epithelium. It provided a barrier to the free movement of molecules and cells as well as minimizing water loss from the underlying tissues. By excreting fibrous molecules, it also plays a role in the formation of the basement membrane (Burgeson and Christiano, 1997).

The hemidesmosome is positioned at the basal membrane of these keratinocytes. The foundation of the hemidesmosome is the transmembrane α6β4 integrin, which have been reported to be necessary for HD formation as HDs are absent in animals null for either subunit (Koster et al, 2003). This particular combination of integrin subunits is specific to mature epithelium (Burgeson and Christiano, 1997). The β4 cytoplasmic domain appears to play the major role in protein recruitment to the HD via the four pairs of fibronectin type III (FNIII) domains and the intervening connecting sequence (CS) in its
cytoplasmic domain. The second FNIII domain is key in the binding to plectin, the third in the interaction with BP180. An additional domain appears to mediate interactions with BP230 (Koster et al, 2003).

Plectin and BP230 are both members of the plectin protein family. Both serve as adapters between the α6β4 integrin and the intermediate filaments. BP230 requires both plectin and BP180 for correct localization (Koster et al, 2003).

BP180 is an additional transmembrane protein that appears to help stabilize the HD that has been seeded with the α6β4 integrin. It may also be able to bind laminin 5 like α6β4 integrin. The current model is an initial interaction between α6β4 integrin and plectin is further organized by the binding of BP180. BP230 is then recruited thereby increasing possible interactions with intermediate filaments in the cytoskeleton. While this model appears to hold true in vitro, the situation in vivo may be a bit more complex as HDs can still form in a plectin-deficient model (Koster et al, 2003).

**Epidermolysis Bullosa Simplex (EBS)**

Abnormal or absent connections within the epidermis between the keratin intermediate filament (IF) network and hemidesmosomes results in EBS and affects about 1 in 25,000 live births worldwide (Coulombe et al. 2009). The characteristic blistering pattern seen in these patients occurs within the layer of the basal keratinocytes of the epidermis and does not lead to disruption of the basement membrane. In contrast to the other EB major subtypes, blisters in EBS heal with no scar due to the favorable plane of separation (Coulombe et al. 2009).
The vast majority of these cases result from mutations in either keratin 5 or 14, a Type I / Type II pair of IFs within the hypodermis (Coulombe et al. 2009). Since these keratins form heterodimers of Type I and Type II keratins, mutations in one can lead to loss-of-function (in the case of null mutants) or dominant negativity (Coulombe et al. 2009), with the phenotype from dominant negative mutations being more severe than the null. The phenotype from these mutations can be modulated by other keratins (such as K15), which can at least partially rescue the effects of such a mutation by replacing the aberrant protein within the heterodimer. Molecularly, the erroneous IF fibers are more fragile than their wild type counterparts and, therefore, fail in response to applied stress.

Mutations in the more severe EBS patients, such as the generalized subtype, have been localized to regions of the gene encoding the helix initiation and termination motifs. Mutations in other areas of the gene, such as that that encodes the linker region, correlate with less severe pathologies. The most severe of these subtypes is EBS with muscular dystrophy (or EBS-MD). Blistering in these individuals was noted by electron microscopic examination to be just within the plasma membrane of the basal keratinocytes. Additionally, muscle cells in these individuals were noted to be disorganized with varying muscle fiber lengths and areas of atrophy and regeneration. This condition, unlike those previously described, is due to mutations in the plectin gene. Plectin (or HD1) is located in the cytosolic (or inner) electron dense region of the hemidesmosome and loss of this protein results in disruption of keratin attachment to the hemidesmosome. Transmission of this condition is autosomal recessive and the blisters
appear in a generalized pattern. Blisters can be present from birth in these patients, but the onset of the muscular dystrophy is often not until infancy or adulthood (Smith et al. 1996).

Kindler Syndrome

First described in 1954 by Theresa Kindler in a 14 year-old patient with congenital blistering on her hands and feet, Kindler Syndrome also results from epidermal layer blistering and is caused by mutations in the KIND1 gene, which encodes the kindling-1 protein. This protein shares homology with the C. elegans protein UNC-112, which has been shown to function in the attachment of actin to the extracellular matrix. Therefore, it has been hypothesized that kindling-1, which has a talin-like domain that could attach to actin and a pleckstrin homology (PH) domain that could bind to phosphatidylinositol phospholipids in the cell membrane, could serve as an actin-membrane link (Seigel et al, 2003).

Kindler Syndrome is very rare, with less than 100 cases in the medical literature, but does have a cluster of patients in a group of Native Americans in Panama. Patients displaying this disease have blistering of the skin from birth to adolescence as well as skin fragility in sun exposed areas and changes in pigmentation (hyper- and hypopigmentation are seen). As adults, the blistering lessens, which has been postulated to indicate that this connection is relied upon more readily by children and that the hemidesmosome-mediated linkage is more robust and able to compensate in adults (Seigel et al, 2003).
Basement Membrane Layer

The basement membrane is a fibrous layer which supports the epidermis, is a scaffold for migrating cells, and controls the passage of molecules and cells to and from the epidermal layer (Burgeson and Christiano, 1997). Laminin 5 (Laminin 332) is the major component of the adhesion complex through the basement membrane. A heterotrimer made up of an α3, a β3, and a γ2 subunit (hence the 332 designation), it is encoded by three subunits, the LAMA3, LAMB3, and LAMC2 genes respectively and are assembled intracellularly prior to secretion (Aumailley and Rousselle, 1999, Jonkman, 1999). Each subunit contains a highly conserved region at the carboxy terminus made of heptad repeats, which in the final molecule combine to form a coiled coil motif. Extending beyond this on the α3 subunit is an additional globular domain termed the LG domain. Together the three subunits form a cruciform shape with the amino termini variable region of each subunit hanging free (Aumailley and Rousselle, 1999).

Laminin 5 localizes to the lamina lucida of the extracellular matrix underlying the epithelia. It serves as a linkage between the α6β4 integrin on the epidermal basal cell membrane and the collagen VII anchored into the lamina densa (Aumailley and Rousselle, 1999). Additionally, laminin 5 associates within the ECM with laminins 6 and 7, which allows it to link into the meshwork ECM network of perlecan and Type IV collagen via nidogen (Jonkman, 1999).

Junctional Epidermolysis Bullosa (JEB)

JEB results from a loss or alteration in the laminin 5 protein within the extracellular matrix (Aumailley and Rousselle, 1999). There are two major types of JEB based on
severity of phenotype and type of mutation seen in the three genes encoding laminin 5, LAMA3, LAMB3, and LAMC2. Both types show autosomal recessive transmission. The more severe type, Herlitz-JEB, is due to a premature stop codon within these genes (Aumailley and Rousselle, 1999). Patients showing this disorder have an abundance of blistering early in infancy and often die secondary to related infections (Fine et al. 2008). Patients with the non-Herlitz, generalized type of JEB, which is due to missense mutations in the laminin 5 genes, are much less likely to die from associated infections before adulthood (Aumailley and Rousselle, 1999, Fine et al. 2008). Therefore, in these cases null mutation is more severe than dominant negative.

There are several minor types of JEB, which are due to mutations in α6β4 integrin and collagen XVII. Mutations in α6β4 integrin lead to JEB with pyloric atresia (JEB-PA). Blisters in JEB-PA also show a generalized distribution, display pyloric atresia, and have multiple other malformations within the GI tract. Death due to EB complications in JEB-PA is equivalent to patients with the Herlitz type of JEB. Associated with mutations in the gene encoding collagen XVII, JEB, non-Herlitz type, localized is the mildest form of JEB described thus far. Blistering is localized to discreet regions in the skin and oral mucosa only, with no other systemic manifestations. Death from localized JEB is rare (Fine et al. 2008).

Dermal Connective Tissue Layer

Underlying the basement membrane, the dermal layer provides additional structural support for the epidermis as well as housing blood vessels and immune cells that further maintain the overlying cells (Goldman and Ausiello, 2008). This are the site of the
anchoring fibrils that complete the anchoring constructs. The major component of these anchoring fibrils, collagen VII, is encoded by the COL4A1 gene. This is an enormous gene spanning 32 kb and containing 118 exons. Each final collagen VII is composed of three α1 subunits, which each contain a central triple helix collagenous domain and two non-collagenous domains, NC1 and NC2, at the amino and carboxy terminus respectively. The triple helix domain contains repeating runs of –Gly-X-Y- with periodic non-collagenous segments interspersed, which are thought to confer a certain amount of flexibility to the molecule. The largest of these segments is a 39 amino acid stretch near the center of the triple helix termed the “hinge region.” Collagen VII is translated as procollagen VII and is post-translationally modified with the cleavage of the NC-2 domain (Bruckner-Tuderman et al. 1999).

The final molecules arrange themselves into fibrils in a tail-to-tail fashion, with the NC-1 domains pointing outwards. With the NC-1 domain, the fibrils attach into either the lamina densa, interacting directly with β3 or γ2 domain of laminin 5, or anchoring plaques in the dermis. As these fibrils hook back to the basement membrane, they trap dermal collagen within these loops, thus providing additional strength to the connections (Bruckner-Tuderman et al. 1999).

**Dystrophic Epidermolysis Bullosa (DEB)**

Dystrophic EB (DEB) is characterized by separation within the dermal connective tissue layer of the skin, below the lamina densa. Most cases of this are due to mutations in the collagen VII encoding gene, COL7A1 (Bruckner-Tuderman et al. 1999). Mutations in COL4A1 can lead to a range of phenotypes from a complete loss of fibrils,
in DEB mutilans, to a relatively simple disordering of the fibrils. Interestingly, a loss of 50% of these fibrils is not sufficient to cause a mutant phenotype. Given that the collagen VII protein is composed of an alpha helical core, one would think that point mutations leading to the replacement of glycine residues with amino acids with larger side groups would have a profound effect on function. Surprisingly, though these would be assumed to disrupt the alpha helices, mild or no mutant phenotype is noted. Heterozygocity for several COL7A1 mutations is often necessary for displaying the most severe phenotypes (Bruckner-Tuderman et al. 1999).

A related disorder to DEB is epidermolysis bullosa acquisita (EBA), which results from an autoimmune reaction with collagen VII. Most of these antibodies are found to react with the NC-1 domain and thus disrupt fibril binding within the lamina densa. Blistering in these patients also results from separation below the level of the lamina densa (Bruckner-Tuderman et al. 1999).

**Studying Adhesion Complexes in *C. elegans***

While many of the vertebrate protein10s and their interactions have been identified, how these connections assemble and correctly localize during development has not been extensively characterized. In order to achieve this, we suggest examining a similar system in the simpler nematode, *Caenorhabditis elegans*. Due to their small size, short generation time, well-characterized development, ease of transgenic animal generation and transparency, C. elegans represents a convenient system for studying cell-matrix connections *in vivo*. Additionally, an extensive amount of genetic information has been gathered on the worm and is available on-line through wormbase.org.
*Caenorhabditis elegans* hypodermal cells contain attachment structures that are functionally analogous to the vertebrate Type I hemidesmosome and are termed fibrous organelles (FOs) (Andrä et al., 1997). Like hemidesmosomes, fibrous organelles are composed of two electron dense regions, approximately 200 nm in diameter, one on the apical side of the hypodermal cell and one on the basal, that are connected by intermediate filaments (Nievers et al., 1999) [Figure 1.2]. The apical-basal distance between these plaques is about 200 nm (Nievers et al., 1999). FOs are positioned within the hypodermis specifically where it overlies the body wall muscles (Hahn and Labouesse, 2001). The role of these FOs is to transmit force generated by body wall muscles across the hypodermis to the overlying cuticle, which serves as the worm's exoskeleton.

Many of the protein components of the FO have been previously identified. These include transmembrane proteins at the basal (LET-805 / myotactin) and apical (MUA-3, MUP-4) hypodermal cell membranes. Myotactin is thought to interact with the ECM via its fibronectin type III repeats, maintaining the link between the hypodermis and the muscle (Hresko et al., 1999). MUA-3 and MUP-4 are thought to provide connections to the cuticle and may be able to interact directly with IFs via their filiggrin-like repeats (Bercher et al., 2001; Hong et al., 2001). The identified adapter protein VAB-10A may serve as a link from the transmembrane proteins to the IFs at both the apical and basal sides of the hypodermis (Michaux et al., 2001). Another protein, VAB-19, has been described as an FO component and though its function has yet to be fully determined, it has been proposed to play a role in actin cytoskeletal organization (Ding et al., 2003). Mutants of myotactin, *vab-19*, and *vab-10a* as well as the intermediate filaments genes
ifb-2 and ifa-3 arrest at the two-fold stage of development and are paralyzed (Ding et al., 2003; Hresko et al., 1999; Woo et al., 2004; Ding et al., 2004), likely due to failure of the mechanical linkage between the body-wall muscles and the cuticle. Mutants in mua-3, mup-4, and mua-6 show muscle separation defects in the larval stages of development.

The Pat Phenotype and Muscles

The search for additional members of the FO developed through a screen for proteins involved in *C. elegans* body wall muscle attachment. The contractile apparatus of *C. elegans* body wall muscle is attached to the extracellular matrix through a focal adhesion complex, which is similar to dense bodies and M-lines in vertebrate muscles. Williams and Waterston sought to determine additional members of this complex after screens for mutations in genes causing abnormal movement and obvious muscle disorganization had reached a point of saturation. In order to identify these extra muscle component proteins, they took advantage of a mutant phenotype noted for muscle-related genes *myo-3, deb-1, and unc-45* (Williams and Waterston, 1994).

In each of these mutants, worms developed normally until the one-and-a-half-fold stage of development (~400 minutes post-fertilization). In wild-type animals, embryos at this stage begin to twitch and then roll vigorously within the egg as the body-wall muscles begin to contract. In the mutants described above, the embryos mildly twitched, failed to roll, and their development was halted at the two-fold stage (~450 minutes post-fertilization) [Figure 1.3]. They termed this the Pat phenotype (Paralyzed and Arrested at Two-fold) (Williams and Waterston, 1994).
Using this phenotype as the target in a screen, Williams and Waterston identified 16 genes, including ten novel genes, named $\text{pat-2}$ through $\text{pat-6}$ and $\text{pat-8}$ through $\text{pat-12}$. Based on the organization of the myosin heavy chain A in their mutants, these genes were classified into four classes, ranging from complete MhcA disorganization in class I to partial organization in class IV (Williams and Waterston, 1994).

Since their original screen, many of the $\text{pat}$ genes have been characterized and noted to be involved in muscle attachment. $\text{pat-2}$ was shown to encode a homolog of $\alpha$-integrin, with $\text{pat-3}$ encoding a corresponding $\beta$-integrin. These serve as the transmembrane link from the muscle contractile apparatus to the overlying extracellular matrix (Williams and Waterston, 1994). $\text{pat-4}$ was shown by MacKinnon et al to correspond to the vertebrate Integrin-Linked Kinase (ILK). MacKinnon et al also produced a kinase-dead $\text{pat-4}$ mutant, which had no abnormal phenotype suggesting that the kinase domain does not play a major role in $\text{C. elegans}$ muscle activity or development (MacKinnon et al, 2002). $\text{pat-6}$ was determined to be a homolog of vertebrate actopaxin, a known focal adhesion protein (Lin et al. 2003).

Mild Pat mutants, such as the $\text{pat-12}$ mutant, were noted to have muscle contractile elements that developed better than in severe Pat mutants, but degenerated secondarily to cell rupture. Also, these animals rolled in their egg prior to their arrest at the two-fold stage. Williams and Waterston noted that both the myosin based A band and actin based I band became partially organized in the $\text{pat-12}$ mutants. In fact, they hypothesized that, since they were unable to definitively determine the age of the arrested mutants, it is possible that those examined represented animals that had were much older that the ~400 minute post-fertilization that a normal Pat would arrest at and that the disorganization of
the muscle contractile elements seen could have been a secondary deterioration. The pat-
12 mutants did bear a striking resemblance to mutants in the emb-9 gene, which encodes
type IV collagen, a basement membrane component. They stated that this would be
consistent with PAT-12 localizing outside of the body wall muscle cell (Williams and
Waterston, 1994).

Because of the subtle differences in its mutant phenotype, we believe that PAT-12 is
not a component of the muscle contractile apparatus like the severe Pats. We hypothesize
that it is a member of the C. elegans fibrous organelle and plays an important role in
maintaining connections between the hypodermis and muscles at the time when muscles
begin contraction. In this way, PAT-12 resembles various hemidesmosomal proteins in
its function. By studying PAT-12 and its role in forming and maintaining the FO, we
hope to learn how a FO organizes and localizes. We hope, then, to apply the lessons
learned from this back to the vertebrate model and see what correlations that can be
drawn to hemidesmosomes.
Figures

Figure 1.1: Diagram of Vertebrate Hemidesmosomes and the Dermal-Epidermal Junction Zone (DEJZ). - The hemidesmosome links epidermal keratins (5 and 14) to laminin 5 in lamina densa. Laminin 5 also links to type IV collagen in lamina densa via laminin 6 and nidogen (not shown). Laminin 5 then binds to anchoring fibrils composed of type VII collagen, which then trap interstitial collagen located in the dermis in their fibers.
Figure 1.2: Diagram of Known Fibrous Organelle Proteins. MUP-4 and MUA-3 act as transmembrane connectors into the overlying cuticle. Myotactin interacts at basal membrane with fibronectin in basement membrane.
Figure 1.3: Comparison of Wild Type Embryonic Development with Pat Development. Development progresses in an identical manner until the two-fold stage of development when the Pat embryos fail to continue elongation and arrest with paralysis. From Williams and Waterston, 1994.
CHAPTER 2: IDENTIFICATION OF T17h7.4 AS pat-12

Introduction and Rationale

In order to determine the sequence of events that result in the formation of the fibrous organelle, we must first know the members of the complex. In order to know characteristics of the member proteins, it is helpful to identify the genes that encode them. We chose to first establish the identity of the gene encoding the PAT-12 protein because of the clear mutant strain available (st430) and its mild Pat phenotype that was different than Pats that are known to be involved in muscle attachment.

Since Williams and Waterston had mapped pat-12 to chromosome III between unc-45 and daf-7 (Williams and Waterston, 1994) [Figure 2.1], we limited our search of candidate genes for pat-12 to this area. Because RNAi experiments are both so easy to perform and since significant amounts of data from high through-put RNAi screens have been widely published, we used this method as a first step of identifying possible pat-12 genes. We took a two-step approach, which took advantage of both of these facts. By first searching available large scale RNAi screens for genes in this region with reported lethal phenotypes for wormbase.org, we could identify potential pat-12 gene candidates. We then could perform our own RNAi experiments on these genes to determine if any of the lethal phenotypes were specifically Pat phenotypes.

After this study, we would expect to have one or more genes that displayed both a reported embryonic lethal phenotype and a Pat phenotype in a significant proportion of the offspring of injected adults in our studies. To prove which of these candidate genes actually corresponds to pat-12, we would then undertake to inject full gene sequences, in
the form of cosmids containing the gene, into \textit{pat-12(st430)} heterozygous adults to check for rescue in their progeny. Although this is a time-consuming process, it would provide definitive results and would be possible if done on only a few candidate genes. Successful rescue by any of these cosmid injections would indicate that the gene contained on that cosmid is \textit{pat-12}.

Once we have the gene identified, we could then seek the mutant that causes the Pat phenotype by full gene sequencing in a \textit{pat-12(st430)} homozygous mutant animal. This would not only confirm our previous results, but might give some indications of a critical region of the gene, as noted with some of the genes noted above, such as the gene encoding keratin 5 and 14. Additionally, we can examine the gene sequence for any clues as to the function of PAT-12 in the form of conserved protein sequences or homologies. In this way, we would form the foundation on which further experiments on the PAT-12 protein could be based.

\textbf{Materials and Methods}

\textbf{Strains Used}

The following \textit{C. elegans} strains were used for the experiments in this chapter: N2 (which is assumed to be wildtype in all experiments), RW1389 [\textit{pat-12(st430)/unc-45(e286)daf-7(e1372)}], WB297 [\textit{pat-12(st430); zpEx311}], and OT170 [\textit{mua-6(nc16); erIs1}]. All \textit{C. elegans} strains were grown on NGM plates seeded with the \textit{Escherichia coli} strain OP50 (Brenner 1974).
Construction of Transgenic Lines

We generated all transgenic lines through microinjection as described by Mello and Fire (Mello and Fire, 1995). In each injection, the plasmid pRF4 [rol-6 (su1006dm)] was used (at approximately 150 ng µl⁻¹) as a marker due to the stereotypic phenotype of a rolling locomotion seen in expressing animals. F1 worms displaying the Rol phenotype were cloned, allowed to self-cross, and the F2 were examined for rolling progeny. All injected lines were considered stable when the balancer phenotype was lost and the roller phenotype was observed in the 2nd generation progeny of injected worms. The extrachromosomal array zpEx311 in the strain WB297 was generated by injection of a solution containing the cosmids F20B8, C24A1, and F42G9 each at concentrations of approximately 10.5 ng µl⁻¹.

RNAi

RNA interference was performed as described by Fire et al (Fire et al., 1998). The cDNA clones yk287e3 and yk15d2, both corresponding to the gene T17H7.4, were amplified independently by PCR using the T7 and T3 primers. Sense and antisense strands of RNA were generated from these PCR products using the MEGAscript T3 and T7 High Yield Transcription Kit (Ambion). The sense and antisense strands corresponding to each cDNA clone were combined, and purified as described by Sambrook and Russell (Sambrook and Russell, 2001), and resuspended in TE pH 8.0. The RNAs corresponding to these two cDNA clones were the combined and injected into N2 (wildtype) hermaphrodites. Only eggs that were laid between 12 and 48 hours after injection were scored for the Pat phenotype.
Sequence Analysis

To determine the genetic lesion in pat-12 allele st430, the following primers, which span the length of the predicted gene T17H7.4, were used: BW-241, BW-242, BW-245, BW-246, BW-249, BW-250, BW-253, BW-254, BW-257, and BW258. Whole N2 (wildtype) worms and 6-8 arrested pat-12 homozygous embryos were incubated in 5 µl of lysis buffer (50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween-20, and 0.01% (w/v) gelatin) with proteinase K (60 µg/ml) at –80°C for 10 minutes, 65°C for 60 minutes, and 95°C for 15 minutes (Williams 1995). PCR fragments were generated using these genomic DNAs by the following protocol: 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 2 minutes 30 seconds, repeated 29 times and held at 4°C. Fragments were prepared for sequencing using the BigDye Terminator Cycle Sequencing Kit (ABI, PE Corporation). Samples were sequenced at the University of Illinois Biotechnology Center. Sequences from Pat embryos were then compared to those of wild-type worms to determine base pair differences.

Results

Molecular Identity of pat-12

We sought to determine the molecular identity of pat-12 through positional cloning in combination with RNA interference (RNAi), transgenic phenotypic rescue, and sequence analysis. Williams and Waterston mapped pat-12 to linkage group III between unc-45 and daf-7 (Williams and Waterston, 1994) [Fig. 2.2A]. This interval contains 52 predicted genes, 7 of which are reported in large-scale screens to display an embryonic
lethal RNAi phenotype. This phenotype was reported most frequently for one predicted
gene in this interval, *T17H7.4* (Rual et al., 2004; Simmer et al., 2003; Sönnichsen et al.,
2005; Tsuboi et al., 2002; Green et al., 2004; Gonczy et al., 2000; Kamath et al., 2003).
Therefore, we repeated the RNAi experiment focusing on this predicted gene as the most
likely candidate. Our RNAi experiments resulted in a 97.8% Pat F1 progeny rate
(n=338), confirming that this embryonic lethal phenotype was a Pat phenotype. This
result established *T17H7.4* as a promising *pat-12* candidate. Because this result was so
positive, we decided to move forward looking only at this gene rather than trying to
identify other possible candidate genes.

To determine directly whether *T17H7.4* is *pat-12*, we attempted to rescue the *st430*
mutant phenotype with the corresponding cosmids. Initially, we focused on three
overlapping cosmids, C24A1, T17H7, and F42G9 (provided by Dr. Alan Coulson). After
we were unable to rescue using these cosmids, we performed restriction enzyme
digestions to determine if they were completely intact. In all of these trials, T17H7
showed a large deletion (data not shown). To overcome this, we used a non-canonical
cosmid, F20B8, which corresponds to T17H7 (also provided by Dr. Alan Coulson).
Digestions of this cosmid showed it to be intact (data not shown).

All three cosmids, C24A1, F20B8, and F42G9 [Figure 2.1 and 2.2B], are required
together because the entire promoter and coding region of *T17H7.4* is approximately 24.7
kb [Fig. 2.2B]. Individually, none of these three cosmids include the entire predicted
*T17H7.4* coding region. We were able to establish a viable transgenic line using an
extrachromosomal array made by co-injecting all three cosmids that rescued the two-fold
arrest of *pat-12(st430)* homozygotes. The individual injection of any one of these
cosmids was insufficient to rescue the \textit{st430} mutation. We were only able to generate one transgenic line from this experiment, likely due to the rarity of \textit{in vivo} crossover events occurring to generate a full length \textit{pat-12} gene with flanking promoter and 3’ untranslated regions. These results are consistent with the identification of \textit{T17H7.4} as \textit{pat-12}.

To further confirm this identification and to attempt to obtain additional information concerning the \textit{pat-12} gene, we looked for the \textit{pat-12(st430)} genetic lesion in \textit{T17H7.4} by DNA sequence analysis. Initially, we used a predicted gene map that included only 3 splice isoforms and lacked the first four exons of isoform K. We isolated the entire predicted coding region of \textit{T17H7.4} at that time by PCR from both wild-type adults and arrested \textit{pat-12} homozygous embryos and compared the DNA sequences. Fortuitously, we found only one change that happened to be in this region, a single guanine to adenine transition in the splice acceptor site of the 12\textsuperscript{th} exon in the \textit{st430} embryos compared to the wild-type [Fig. 2.2C]. This mutation alters the highly conserved AG splice acceptor sequence (Ding et al., 2003; Blumenthal and Steward, 1997) [Fig. 2.3] and most likely would result in the loss of exon 12. Of the 12 current predicted isoforms of \textit{T17H7.4} from wormbase.org, exon 12 is included in only four: isoforms A, C, J and K [Fig. 2.1C]. Our results, therefore, confirm that \textit{T17H7.4} is \textit{pat-12}, and further suggest that a deficiency in one or more of these four PAT-12 proteins is responsible for the muscle detachment and subsequent developmental arrest in \textit{pat-12(st430)} mutants. They also suggested exon 12 as a possible critical region to further investigate by examining sequence homologies with vertebrate genes.
Discussion

The original description of the *pat-12* “mild” Pat phenotype suggests that this gene is required at some level for the stable attachment of body wall muscle cells to the hypodermis during embryogenesis. Because its mutant phenotype is so different than that of previously identified Pat genes that are known to encode muscle attachment proteins, we suspected that PAT-12 did not localize to the focal adhesion complexes. Additionally, Williams and Waterston hypothesized that since a severe disruption of the muscle contractile apparatus was not seen until after the stage of arrest, it was probably not an intricate part of the muscle assembly, and may be acting in the hypodermis (Williams and Waterston, 1994). We have shown here that the genetically defined *pat-12* locus corresponds to the proposed gene *T17H7.4* by RNAi, genetic rescue, and mutation identification.

The proposed gene *T17H7.4* encodes 12 predicted isoforms and extends over about 25 kb of chromosome III [Fig. 2.2C]. Examination of the sequences of all 12 predicted isoforms of *T17H7.4* and their protein products revealed no conserved domains, NLS, NES, or transmembrane sequences, suggesting that all are cytosolic proteins. No areas of conserved domains were noted in the exon (exon 12), which we predict would be lost in the *pat-12(st430)* homozygous mutant. The four predicted isoforms, A, C, J, and K, likely affected by the *st430* mutation encode proteins of 663, 706, 716, and 657 amino acids respectively.

BLAST searches of these protein sequences identified only three closely related proteins, OvB20 in Onchocerca volvulus and B20-1 and B20-2 in Brugia malayi, but no clear vertebrate homologs. OvB20 is a protein characterized in larva of Onchocerca
volvulus, a cause of onchocerciasis in cattle and has been studied as a possible target for immunization. It was shown to localize to discrete area in the hypodermis and cuticle of larvae. Vaccination against this protein offered protection from onchocerciasis in rodents (Taylor et al., 1995).

The specific mutation in the st430 mutant, the G to A mutation, is at a highly conserved splice acceptor site of exon 12. Blumenthal and Steward note that this site is a CAG in 85% of introns that were examined in C. elegans (Blumenthal and Steward, 1997). Aroian et al., however, describe mutations in three genes, let-23, dpy-10, and let-23, in which a similar G to A mutation at the splice acceptor site did yield some correctly spliced RNA copies. Each of these introns was small [53, 48, and 316 nt respectively], whereas the intron proceeding exon 12 is about 800 nt in length. The authors here suggest that larger introns could be spliced to a mutant AA sequence or a nearby AG, but it is clear from their data that other transcripts will be present in significant quantities (Aroian et al, 1993).

If some of the original transcript can be made and spliced like the wild type, the question becomes: why does this not rescue the mutant phenotype. The first possibility could be a dosing issue. Aroian et al. stated that in the case of the genes they examined, only about 52% of the transcripts were approximately the same length as the wild type. This allowed for the use of aberrant splice site near the proper site (Aroian et al, 1993). If there is a certain threshold amount of PAT-12 necessary for muscle attachment at the two-fold stage, this lower amount may not be enough to function.

Secondly, the improperly spliced transcripts may be exerting a dominant negative effect on the wild type copies. This would be more likely the case if it appeared the
PAT-12 was playing a signaling role and not a structural role as we propose. If this were, in fact, the case, rescuing the mutants with any construct that is not a major over expression of \textit{pat-12} would be very difficult or impossible. Further studies localizing PAT-12 in wildtype and mutant animals are needed to differentiate these. We will describe these experiments in the next chapter.
Figures

Figure 2.1: Physical Map of Area Between *unc-45* and *daf-7* from Chromosome III with Cosmids Contained Therein. - Cosmids containing *unc-45* and *daf-7* are in red. Cosmids injected for rescue of the *pat-12(st430)* mutation or the canonical cosmids representing them [T17H7 is the canonical cosmid for F20B8] are shown in yellow.
Figure 2.2: Gene Map of *pat-12* from Chromosome III. (A) *pat-12* is located in a 300 kb region between *unc-45* and *daf-7*. (B) Three overlapping cosmids together contain the coding region and 5' UTR of *pat-12*, F42G9, F20B8, and C24A1. (C) 12 potential isoforms of *pat-12* have been predicted. The area of the G to A mutation in the *st430* mutants is highlighted. Isoforms predicted to be affected by the *st430* mutation (J, A, C, and K) are marked in blue. The star indicates the region of *pat-12* that is targeted by the anti-*pat-12* antibody.
**Figure 2.3: C. elegans Consensus Sequence of Splice Donor and Splice Acceptor Site for Cis-splicing** - Both 5’ and 3’ site are with respect to intron [5’ is splice donator, 3’ is splice acceptor]. Consensus sequence of CAG is present at splice acceptor site in 85% of introns examined. The *pat-12(st430)* mutation is a G to A mutation in the –1 position of the 3’ splice site. Adapted from Blumenthal and Steward, 1997.

<table>
<thead>
<tr>
<th>5’ Splice Site</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>+5</th>
<th>+6</th>
<th>+7</th>
<th>+8</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>16</td>
<td>12</td>
<td>64</td>
<td>100</td>
<td>0</td>
<td>24</td>
<td>9</td>
<td>76</td>
<td>7</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>A</td>
<td>42</td>
<td>56</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>59</td>
<td>67</td>
<td>10</td>
<td>19</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>U</td>
<td>16</td>
<td>18</td>
<td>12</td>
<td>0</td>
<td>100</td>
<td>16</td>
<td>17</td>
<td>10</td>
<td>62</td>
<td>54</td>
<td>52</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>14</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3’ Splice Site</th>
<th>-7</th>
<th>-6</th>
<th>-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>37</td>
</tr>
<tr>
<td>A</td>
<td>31</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>U</td>
<td>53</td>
<td>89</td>
<td>98</td>
<td>70</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>15</td>
<td>83</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>
CHAPTER 3: PAT-12 PROTEIN

Introduction and Rationale

With the identity of *pat-12* now known as *T17H7.4*, we turned our attention to the attributes of the PAT-12 protein. The only additional information that we gained from examination of the sequence of PAT-12 was that it is a cytosolic protein since it lacked a transmembrane domain. With no further clues to its function, we now worked to elucidate this by examining its expression and specific sub-cellular localization.

The first question that needed to be addressed was which isoform or isoforms, if any, are present in the hypodermal cells and thus likely involved in the fibrous organelle. We chose to approach this by a two-fold method of generating an anti-PAT-12 antibody and transcriptional fusion products corresponding to various isoforms. The first of these methods offers a reagent that will be invaluable for future experiments and can also be used to determine sub-cellular localization, but does not differentiate between isoforms that contain the sequence used as an antigen. While the second gives information on which isoforms are expressed by the hypodermal cell, provided they use different promoter regions, it does not allow for the precise protein localization. Therefore, we needed the two in conjunction to delineate which isoforms on which to focus future experiments. Additionally, we chose these two specific methods to determine this because of their success in experiments on previous Pat proteins (PAT-4, PAT6) (MacKinnon et al, 2002, Lin et al, 2003).

To determine the specific sub-cellular localization of PAT-12 we have elected to once again use our new anti-PAT-12 antibody, supplementing it with translational fusion
products (containing gfp in frame) corresponding to the hypodermally expressed isoforms previously noted. Once again, these two lines of experimentation compliment each other. By staining animals with our antibody and those directed at previously identified fibrous organelle proteins, we can determine if PAT-12 localizes with this structure. Also, the reagent affords us the opportunity to stain pat-12(st430) homozygous mutant animals to elucidate whether any PAT-12 protein is present in these animals. The translational fusion constructs will allow us to confirm antibody data and also view PAT-12 localization in a live animal.

Furthermore, we could look at PAT-12::GFP expressing animals over the course of their development to see if expression of different isoforms changes. As noted in chapter one, members of hemidesmosome related adhesion complexes, such as keratins, are replaced by other similar proteins during development. In this way PAT-12 could be lending some flexibility to the fibrous organelle in response to environmental alterations. Ultimately, we desire to place PAT-12 within the fibrous organelle and, building on previous work of other member protein, create a foundation on which experiments to determine the sequence of events that mark the FO formation.

Materials and Methods

Strains Used

The following C. elegans strains were used for the experiments in this chapter: N2 (which is assumed to be wildtype in all experiments), RW1389 [pat-12(st430)/unc-
45(e286)daf-7(e1372)], WB297 [pat-12(st430); zpEx311], and OT170 [mua-6(nc16); erIs1]. All C. elegans strains were grown on NGM plates seeded with the Escherichia coli strain OP50 (Brenner 1974).

Construction of Transgenic Lines

We generated all transgenic lines through microinjection as described by Mello and Fire (Mello and Fire, 1995). In each injection, the plasmid pRF4 [rol-6 (su1006dm)] was used (at approximately 150 ng µl⁻¹) as a marker due to the stereotypic phenotype of a rolling locomotion seen in expressing animals. F1 worms displaying the Rol phenotype were cloned, allowed to self-cross, and the F2 were examined for rolling progeny. All injected lines were considered stable when the balancer phenotype was lost and the roller phenotype was observed in the 2nd generation progeny of injected worms.

Lines expressing transcriptional fusion constructs were generated by injecting plasmids that contained genomic sequence upstream from the start site of each isoform (amplified from the cosmid F42G9 [provided by Dr. Alan Coulson, Wellcome Trust Sanger Centre]) fused to the coding region of the gfp gene (amplified from the cosmid pPD118.20 [provided by Dr. Andrew Fire, Carnegie Institute]). Each was injected into a wild-type N2 background at concentrations of 5 – 10 ng µl⁻¹. The extrachromosomal arrays zpEx312 in the strain WB298 was generated by the injection of pJMR260. This array contains the 2.389 kb genomic region upstream from the start of the K isoform of T17H7.4 fused to gfp. Similarly, the extrachromosomal array zpEx313 in the strain WB299 was generated by the injection of pJMR255. This array contains the 2.575 kb genomic region upstream from the start of the A and C isoforms of T17H7.4 fused to gfp.
The extrachromosomal array zpEx314 in the strain WB300 was generated by the injection of pJMR252. This array contains the 2.463 kb genomic region upstream from the start of the J isoform of T17H7.4 fused to gfp.

Lines expressing translational fusion constructs were generated by injecting plasmids that contained genomic sequence upstream from the start site (amplified from the cosmid F42G9 [provided by Dr. Alan Coulson, Wellcome Trust Sanger Centre]) and the coding region of each isoform (amplified from the cDNAs yk1043e9 (Isoform K) and yk287e3 (Isoform C) [provided by Dr. Yuji Kohara, National Institute of Genetics, Japan] fused to the coding region of the gfp gene (amplified from the cosmid pPD118.20 [provided by Dr. Andrew Fire, Carnegie Institute]). Each was injected into pat-12(st430) heterozygotes. The extrachromosomal arrays zpEx326 [Isoform K::GFP] in the strain WB326 was generated by the injection of two overlapping PCR products generated using the template pJMR266 with the primers WB-615 and WB-616 and the template pJMR300 with the primers BW-617 and BW-618. These PCR products were injected at 10 ng µl⁻¹. Similarly, the extrachromosomal array zpEx327 [Isoform C::GFP] in the strain WB327 was generated by the injection of a solution containing the overlapping PCR products generated using the template pJMR293 with the primers BW-615 and BW-616 and the template pJMR400 with the primers BW-617 and BW-618. These were injected at 10 ng µl⁻¹.

**Antibody Staining**

All embryos were fixed stained as described by Barstead and Waterston (Barstead and Waterston, 1991). Embryos were released from gravid adults by treatment with basic
hypochlorite solution (0.5 N KOH, 1.2% NaOCl) for 7 minutes and washed four times in M9 buffer solution (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1mM MgSO₄) (Sulston and Hodgkins, 1988). Embryos were fixed in a formaldehyde fixative (3% formaldehyde from paraformaldehyde, 0.1 M sodium phosphate, pH, 7.0, 0.1 mM EDTA) for 10 minutes at room temperature and washed two times with PBS, pH 7.0. They were then incubated -20°C methanol for 10 minutes at –4°C. They were then washed two times in PBS pH 7.0. Embryos were then incubated two times in PBS, pH 7.0, containing 0.5% Tween-20. Embryos were then incubated for 45 minutes in the primary antibody diluted in 70% PBST (PBS, pH 7.0, 0.5% Tween-20) and 30% normal goat serum and washed four times in PBST for 5 minutes. Embryos were then incubated in secondary antibody diluted in 70% PBST (PBS, pH 7.0, 0.5% Tween-20) and 30% normal goat serum and washed four times in PBST for 5 minutes. Embryos were stored in 50% PBS, pH 7.0 with 50% glycerol. Embryos were then mounted and viewed.

Primary antibodies were diluted as follows in 250 ml of 70% PBST (PBS, pH 7.0, 0.5% Tween-20) and 30% normal goat serum: MH4 [anti-ifa-1, 2, and 3] – 1:100, MH46 [anti-myotactin] – 1:100 [provided by the Developmental Studies Hybridoma Bank], and anti-PAT-12 – 1:2000. In each experiment, a goat anti-rat fluorescence conjugated secondary antibody was used, diluted 1:100 in 70% PBST (PBS, pH 7.0, 0.5% Tween-20) and 30% normal goat serum.

Antibody Generation

PAT-12 antisera was obtained by injecting mice with a synthetic peptide [N-CRRNLAGDFPSEHSKSKIKLDPLEQR] fused to KLH [Immunological Resource
Center, University of Illinois]. This sequence is shared by the A, B, C, and K isoforms of T17H7.4. Polyclonal sera from injected mice were used to stain N2 (wild type) worms as described above.

**Determination of Developmental Stages**

The developmental stages of embryos and larva was determined by allowing 10 adult worms to lay eggs on a new plate for 1 hour, removing the adults, and allowing the progeny to develop for the time specified for each stage as stated by Wood (Wood, 1988). Specifically, the embryonic stages were defined as from 0-12 hours post laying, the L1 larval stage from 12-23 hours post laying, the L2 larval stage from 23-30 hour post laying, the L3 larval stage from 30-38 hours post laying, the L4 larval stage from 38-47 hours post laying, and the adult stage after 47 hours post laying.

**DiI Staining of Amphid and Phasmid**

WB326 hermaphrodites were incubated in DiI (2 mg / ml in dimethyl formaldehyde) diluted 1:1500 in M9 for either 30 minutes (for amphid visualization) or 2 hours (for phasmid visualization). Worms were plated to NGM plates seeded with OP50 *E. coli* for 1 hour to destain and then visualized (Uchida et al, 2003).
Results

Isoforms A, C, and K are Expressed in the Hypodermis

To further investigate the cause of the \textit{pat-12(st430)} muscle detachment and developmental arrest phenotype, we sought to determine the expression pattern of the protein products of the A, C, J and K isoforms, the protein products of the isoforms affected by the mutation in \textit{pat-12(st430)} mutant worms. As shown in Figure 1C, isoforms J and K have distinct transcriptional start sites, while isoforms A and C share a common start site. We, therefore, generated three GFP transcriptional reporter constructs each of which included 2.3-2.5 kb of DNA upstream from the predicted start of translation. In other fibrous organelle protein studies, similar length promoter sequences have been sufficient to drive expression (Ding et al, 2003, Karabinos et al, 2001). The constructs were introduced into wild-type worms though standard transformation techniques.

The reporter for isoforms A and C showed GFP expression in the hypodermis [Figs. 2C, D] as well as in epithelial cells of the rectum, vulva, amphids and phasmids [data not shown]. The isoform K reporter showed an identical pattern of expression [Figs. 2E, F]. Interestingly, the isoform J reporter showed GFP expression in the pharynx and the rectal epithelium [data not shown], but not in the hypodermis [Figs. 2A, B]. Given these expression patterns, we hypothesize that the muscle detachment phenotype in \textit{st430} mutants is likely due to the lack of one or more of the isoforms A, C, and K in the hypodermis. We, therefore, focused our investigation on the localization of isoforms A, C, and K within the hypodermis.
Anti-PAT-12 Antibody Staining

To confirm translational fusion construct data and to determine the localization of the protein products of isoforms A, C and K in vivo, we generated polyclonal antibodies against a synthetic peptide corresponding to a region of the 5th – 8th exons [Star in Fig. 1C], which is shared by the A, C, J, and K as well as the B isoforms. Staining of wild-type N2 worms and embryos revealed a circumferential hypodermal banding pattern that sub-tends the sites of body-wall muscle attachments [Fig. 3A, B] and appears identical to the patterns reported previously for other fibrous organelle proteins (Hresko et al, 1999). In addition, we observed pharyngeal staining.

To determine localization of PAT-12 isoforms in pat-12(st430) arrested embryos, we stained arrested embryos with the polyclonal antiserum. The hypodermal cells fail to stain in the arrested mutant animals [Fig. 3F], while the pharyngeal staining remains [Fig. 3E]. In comparable staged wild-type control embryos, both the hypodermal [Fig. 3D] and pharyngeal staining patterns are visible [Fig. 3C]. These results, in combination with the transcriptional reporter results above, suggest that the st430 mutation blocks the expression of isoforms A, C, and K from the hypodermis, but does not effect the expression of additional pharyngeal isoform(s). The pharyngeal signal is likely to come from isoform B, which should also be detected by the polyclonal antiserum and would not be affected by mutations to exon 12. Taking all the results together, it is likely that the loss of one or more of the three isoforms A, C, and K from the hypodermis results in the muscle detachment and developmental arrest in the pat-12(st430) mutants.
Rescue By and Location of Isoforms C and K in vivo

To test this idea more directly, we examined whether any one of these isoforms individually is sufficient to rescue the *pat-12(st430)* homozygotes from developmental arrest. We, therefore, sought to generate plasmids containing mini-genes, each of which would include a single isoform fused in frame to *gfp*. This was attempted multiple times with similar results on every occasion, the resulting plasmid showed poor transformation rates into the *E. coli* and contained new mutations not present in the original cosmid. This, along with the previously reported large deletion in the cosmid T17H7, led us to speculate that the *pat-12* gene is lethal to *E. coli* and thus acts by natural selection to promote those colonies that receive constructs whose inserts are mutated during the process of PCR. Knowing this, we had to resort to injecting two overlapping PCR fragments for each isoform, one containing the promoter, one containing the coding region and one containing *gfp*. While this was successful only on rare occasions, we were able to generate transgenic animals.

We tested these transgenic mini-genes encoding either isoform C or K tagged with GFP and found that both on their own were sufficient to rescue the *st430* embryonic arrest phenotype. In contrast, repeated attempts to rescue *pat-12(st430)* mutants with an isoform A minigene failed to rescue the developmental arrest. Our results suggest that it is the loss of both C and K together that leads to *pat-12* developmental arrest, and that these two isoforms have at least partially overlapping functions.

The C isoform mini-gene was expressed in the hypodermis [Fig. 4F], in an extension from the sieve to the grinder in the pharynx [Fig. 4H], and in the rectal epithelia [Fig. 4G]. The expression of the C isoform mini-gene was highest at the early L1 larval stage.
[Figs. 5A, B]. This expression level stayed high through the L3 larval stage [Figs. 5I, J], decreased through the L4 larval stage [Figs. 5M, N], and was greatly diminished by adulthood.

The K isoform mini-gene was expressed in the hypodermis [Fig. 4A], vulva [Fig. 4D], rectal epithelia [Fig. 4E], amphids [Fig. 4B], and phasmids [Fig. 4C]. To confirm the amphid and phasmid localization of isoform K, we incubated transgenic worms in DiI, which is taken up into both amphids and phasmids. In both locations, the GFP signal localized to structure stained with the DiI [Data not shown]. The expression of the K isoform mini-gene was not visible until the late L1 larval stage [Figs. 5A, B]. Expression levels then started to increase throughout larval development [Figs. 5C’, D’, E’, F’, G’, and H’] and were highest in the adult worms.

**Comparison of PAT-12 and FO Protein Localization**

We have hypothesized that PAT-12 localizes to the *C. elegans* fibrous organelle due to its characteristic hypodermal banding pattern [Figs. 3A, B]. To show this directly, we stained worms expressing the GFP-tagged isoform K with antibodies against the known fibrous organelle components myotactin and the intermediate filament protein MH4. As we expected, the GFP-tagged PAT-12 isoform K co-localized to circumferential bands in the hypodermis with both MH4 and myotactin [Fig. 6] signifying that PAT-12 most likely is an FO component.
Discussion

Our expression data and mini-gene rescue experiments show that three of the PAT-12 isoforms are located in the FOs of hypodermal cells, which are organized specifically at locations overlying muscle cell attachments. Our mini-gene rescue experiments suggest that the simultaneous loss of both isoforms C and K causes developmental arrest. Isoform C is expressed at early stages of larval development with expression decreasing in late larval stages and adulthood. Isoform K begins to be expressed at the L2 stage and persists through adulthood. This suggests that while these isoforms likely overlap in function, they are not identical. Also, since transgenic lines expressing only one isoform were not healthy, both isoforms likely perform individual functions at specific times during development.

PAT-12 is a Component of the Fibrous Organelle

We have shown both by antibody staining and with GFP-tagged PAT-12 fusion proteins expressed in vivo that PAT-12 isoforms C and K are components of the FO due to their circumferential banding pattern, which is similar to that seen in other FO proteins. Co-localization studies with the anti-IF antibody MH4 showed that in isoform K these circumferential bands were coincident with the FO intermediate filaments. Recent high through-put binding studies have shown that PAT-12 binds to the intermediate filament IFA-2 in both yeast-2-hybrid and immunoprecipitation studies (Li et al, 2004). These data, taken together, suggest highly that PAT-12 is a component of the fibrous organelle.
**PAT-12 Expression Changes Throughout Development**

The translational fusion products of isoforms C and K both showed significant changes in expression throughout larval development. While C was expressed highly at the L1 larval stage and decreased by the L4 larval stage, the K isoform showed low expression until the L4 larval stage and then continued to be highly expressed in the adult worms. Other FO proteins showed similar changes in expression throughout development, suggesting that some remodeling of the fibrous organelle may occur over the time course of development (Hapiak et al, 2003). Interestingly, since no GFP signal could be visualized at that stage in our transgenic lines, it appeared that neither the C nor the K isoform was highly expressed at the two-fold stage of development when the *pat-12(st430)* mutant embryos arrested. However, both were sufficient to fully rescue the Pat phenotype of these arrested embryos. This would suggest that such low levels of PAT-12 are required to rescue the mutant worms and allow for embryonic development to proceed that we were unable to visualize the GFP attached to them.
Figure 3.1: Expression Patterns of the Transcriptional Fusion Products of the Predicted J, A/C, and K Isoforms of *pat-12* in Adult Worms. - The 5’ promoter region of each predicted isoform was fused to the coding region of the *gfp* gene to make isoform-specific transcriptional fusion products. These were then injected into wild-type N2 hermaphrodites. Isoforms A and C share a common start site and therefore only one transcriptional fusion product was made corresponding to both of these isoforms. The transcriptional fusion products of both isoforms K (C and D) and A/C (E and F) were expressed in hypodermal cells in adults. (E and F) The transcriptional fusion product corresponding to predicted isoform J (A and B) was not expressed in the hypodermal cells in adults. The scale bars represent 15 µm.
Figure 3.2: Staining of Wild Type Adult Worms, Wild Type Embryos, and *pat-12* (*st430*) Mutant Embryos with Anti-PAT-12 Antibody. This antibody was generated to a region of *pat-12* common to all predicted isoforms that are affected by the *st430* mutation (J, A, C, and K) as well as the B isoform. In wild-type adults, the circumferential banding pattern in four quadrants was seen, which is similar to that described for other fibrous organelle proteins (A and B). This banding pattern was also seen in wild-type embryos (D) along with a pharyngeal pattern (C). In the *st430* mutant embryos, the hypodermal circumferential banding pattern was lost (F), but the pharyngeal pattern remained (E). The scale bars represent 15 µm.
Figure 3.3: Co-localization of the *pat-12* Isoform K Translational Fusion Product and Antibodies Against the Fibrous Organelle Proteins myotactin and MH4. - Adults expressing the *gfp*-tagged translational fusion product corresponding to isoform K were stained with antibodies against myotactin [MH46] and the intermediate filament protein MH4. Isoform K (A and D) co-localized with both MH46 [MH46 alone (B), composite (C)] and MH4 [MH4 alone (E), composite (F)] in a hypodermal circumferential banding. The scale bars represent 15 μm.
Figure 3.4: Expression of *pat-12* Isoform Specific Translational Fusion Products in Rescued *st430* Homozygous Mutant Adults. - The entire coding region of isoforms C and K were fused in frame to the *gfp* reporter gene and injected into *st430* heterozygotes. Rescued progeny were cloned and examined as adults for GFP localization. Isoform K was expressed in a circumferential banding pattern in four quadrants (A) that is similar to that described for fibrous organelle proteins. Isoform K was also expressed in the amphid (B), phasmid (C), vulva (D), and rectal epithelium (E). Likewise, isoform C showed a similar hypodermal banding pattern (F). Additionally, isoform C was expressed in rectal epithelium (G) and the pharynx (H). The scale bars represent 15 µm.
Figure 3.5: Expression of *pat-12* Isoform Specific Translational Fusion Products at Different Stages of Larval and Adult Development. - Rescued worms expressing the *gfp* tagged translational fusion product corresponding to isoform K were examined for hypodermal expression through the four larval stages. Isoform C was expressed during the L1 (A and B), L2 (E and F), and L3 stage (I and J) and decreased in the L4 stage (M and N). Expression of isoform K was minimal during the L1 stage (C and D). Expression was maintained at high levels throughout the rest of larval development [L2 (G and H), L3 (K and L), L4 (O and P)]. The scale bars represent 15 µm.
CHAPTER 4: DISCUSSION

Summary

In order to enable us to use a nematode model of hemidesmosome formation, we sought to identify another member of the *C. elegans* hemidesmosome-like fibrous organelle complex. We focused on a mutant strain of *C. elegans* that was isolated due to a developmental arrest phenotype, the Pat phenotype. The mutation in this line had previously been mapped to an area of linkage group III between *unc-45* and *daf-7*. After finding a predicted gene in this area that displayed a Pat phenotype when exposed to the corresponding ssRNA, we located a mutation in this gene, *T17H7.4*, which would lead to a G to A mutation in a splice acceptor site likely resulting in the loss of the encoded protein. Injection of DNA corresponding to this predicted gene rescued the *pat-12(st430)* mutants, thereby indicating that *T17H7.4* is *pat-12*.

Once we had identified the genetic identity of *pat-12*, we injected transcriptional fusion constructs corresponding to the isoforms affected by the *st430* mutation into wild type worms to determine which cells expressed *pat-12* and which isoforms were expressed in the hypodermis. After finding that isoforms A, C, and K were expressed in the hypodermis, our tissue of interest, we desired to know if any of these localized to a pattern similar to fibrous organelle proteins.

Using an antibody to all of these isoforms as well as isoforms J and B, we determined that at least one isoform did localize to the stereotypical circumferential bands. By injecting translational fusion constructs corresponding to isoforms C and K, we found that both of these isoforms displayed the desired localization. We were not able to
generate a translational fusion isoform corresponding to isoform A. Additionally, we noted that these isoforms co-localized with known fibrous organelle proteins. By examining animals expressing these constructs over time, we saw that both isoforms showed changing expression levels over the course of development, specifically that the K isoform was expressed later in development than the C isoform.

Summary of PATc12 Paper by Hetherington et al

As we were compiling the results described above, Hetherington et al. reported the results of their examination of PAT-12. They focused on PAT-12 as a potential target for drugs or vaccines to limit nematode infection as PAT-12 showed homology to another vaccine target, B20 in *Onchocerca volvulus*, as described above (Taylor et al, 1995). They chose to focus on the mutant *pat-12(tm2298)*, which contains a large deletion and which they claim has a more severe phenotype than the *st430* mutant. By both RNAi against *T17H7.4* and by sequencing the *tm2298* mutant, they determined, as we did, that *T17H7.4* corresponds to *pat-12*.

Similarly to what we had done, Hetherington et al. generated and injected translational fusion constructs into mutant *pat-12* worms corresponding to isoforms J, A, K, and E. They stated that both isoforms A and K were expressed in the hypodermis and localized to fibrous organelle component-like circumferential bands, but only isoform A was able to rescue the *tm2298* mutant. When they went on to examine the mutant worms by electron microscope, they found that at the two-fold stage, myotactin remained at the basal membrane and MUP-4 remained associated with the apical membrane in the progeny of wild-type worms fed ssRNA corresponding to *pat-12*. They did, however, see...
a widening of the apical-basal hypodermal distance compared to wild type. Interestingly, along with this widening, they saw detachment of body wall muscle from the hypodermis in the head and tail region of *pat-12* mutant worms (Hetherington et al, 2011).

Through a yeast-2-hybrid screen, they confirmed previous reports that an N-terminal region of PAT-12 bound VAB-10. When they examined the apical-basal hypodermal distance in VAB-10 depleted worms, they reported the same hypodermal widening. In light of this, Hetherington et al. suggest that PAT-12 acts as an adapter interacting with VAB-10 both at the apical and basal membranes of the hypodermal cell to stabilize the fibrous organelle and state that the apical and basal functions of PAT-12 might differ (Hetherington et al, 2011).

While many of our results were similar, there were key differences. First, we were able to rescue the *pat-12(st430)* mutation using isoform K, which they were unable to do. Given our previously described problems generating constructs due to *pat-12* seemingly being incompatible with bacterial growth, there may have simply been some problem with their construct which, though it allowed for proper localization, made rescue impossible. Second, while they stated that isoform A localized to the hypodermis and not isoform C, we found that isoform C not only localized here, but also could rescue the mutants. Since isoforms A and C differ only in one exon in the middle of the gene (Isoform C has the exon, isoform A lacks it), it is possible that both our constructs targeted the same protein. Potentially, this exon could cause very little difference in the final protein (Hetherington et al, 2011).

In these ways, the data that we generated and the data generated by Hetherington et al. complement each other, suggesting that PAT-12 plays a complex and possibly
multiple roles in FOs at the apical and basal membrane. The differences in our results suggest that predicted isoform data may not be entirely correct. Additionally, as they did not mention having difficulty amplifying DNA corresponding to \textit{pat-12} in bacteria, it could be that the differences in the ability to rescue of different isoforms may be due to mutations introduced into their constructs at this point in the experiment. Sequencing of their constructs was not available for verification, but would be helpful before taking either groups results to be fully accurate.

**Predicted Interactions and Model of PAT-12 Function**

Additional information regarding the role of PAT-12 within the fibrous organelle could be gained by examining its proposed or described \textit{in vitro} interactions. Yeast-2-hybrid results suggest that PAT-12 might function in a link between fibrous organelles and actin based circumferential microfilament bundles (CFBs). PAT-12 has been reported to interact with IFA-1, MUA-6 / IFA-2, and IFB-2, which are intermediate filament proteins (Li et al., 2004, Zhang and Sternberg, 2006). Furthermore, PAT-12 was reported to interact with the protein product of the predicted gene \textit{ZK849.2}, which, in turn, was found to interact with VAB-19, a proposed actin interactor, which displays a Pat phenotype and is both dependent on myotactin for proper localization and necessary for proper myotactin localization (Li et al., 2004; Ding et al., 2003, Zhang and Sternberg, 2006).

Actin-based CFBs form rings within the hypodermal cells and function during embryonic elongation to transform the bean shaped embryo into a worm shaped larva without cell divisions by contraction of the hypodermal cells (Costa et al., 1997). As
these bundles contract, the short axis of the hypodermal cells is squeezed forcing expansion of the long axis. Studies have shown that these circumferential bundles attach to an exoskeletal element called the embryonic sheath, which forms the outer layer of the cuticle in the adult (Costa et al., 1997) and localize to areas that will be the furrows between annuli in the adult. When embryonic elongation is complete, these fibers destabilize, but reform during larval molting events (Costa et al., 1997).

Given the proposed interactions with both intermediate filaments and the possible actin-associated protein VAB-19, through ZK849.2, we propose that PAT-12 forms part of a critical link between FOs and CFBs [Figure 4.1]. During times during development when the cuticle is not present, muscles generate forces generated that cannot be transduced to the cuticle by the FOs. At the two-fold stage of development, for example, the cuticle has not formed and therefore, it is unlikely that MUA-3 and MUP-4 can link the IFs to any exoskeletal structure. *pat-12* mutants, therefore, would arrest at this two-fold stage because the loss of the PAT-12 protein leads to the uncoupling of these two structures and allows the muscle cells to disassociate from the hypodermal cells when the muscles begin to contract.

This explains a finding that Hetherington et al. described in which muscle detachment at the two-fold stage in *pat-12* mutants was largely confined to areas at the head and tail of the worm and at the bend in the embryo (Hetherington et al, 2011). In an average hypodermal cell along the length of the embryo, forces generated by body wall muscles on one side of the cell offset forces generated by cells on the other side, resulting in a cumulative force vector equal to zero [Figure 4.2B]. At the head and tail, there are no muscle cells on the other side and thus there is a resultant force vector towards the middle
of the animal [Figure 4.2C]. Likewise, at the bend in the embryo, the forces are not 180
degrees offset from each other and thus there is a resultant force vector away from the
hypodermal cell [Figure 4.2D]. In each of these cases, we propose that without the
cuticle to transmit forces to, the attachment between muscles and the hypodermis relies
on a linkage to the CFBs to maintain integrity in areas where forces are not offset. As
this link is absent in *pat-12* mutants, the muscle cells dissociate.

During later developmental stages, this IF-actin linkage complex could serve to link
the IFs to other actin based structures. This could serve to stabilize and transduce muscle
forces across the entire hypodermal cell via stress fibers. Additionally, at times of
molting, the FO-CFB link would be necessary as the cuticle is not present. This is
supported by the fact that the CFB reforms during these times (Costa et al., 1997).
Differential binding could be achieved through the expression of different isoforms of
PAT-12 at different developmental stages as shown with the two isoforms described
above.

**Comparisons with Vertebrate Hemidesmosomes**

If one is to use the *C. elegans* fibrous organelle as a model to study hemidesmosomal
organization, one needs first see if they are indeed comparable. That is, can information
from studying how the fibrous organelle forms be used to understand vertebrate
hemidesmosomes. First, though they do not contain homologous proteins, the two
structures do share a function, that is to transmit forces, generated either internally or
externally, to a cytoskeletal component. Next, when each structure forms, the basally
situated transmembrane protein, myotactin or integrin, acts to organize and localize the
structure. From this one could suggest that other steps within the organization of these structures are also very similar.

Also, in both structures, as PAT-12 is a testament to, the expression of component proteins changes over time. This is also evident in the switch in expression of keratins during the maturation and migration of epidermal cells to the skin surface. In both cases, this shows that these two are dynamic structures, able to adapt to changing intracellular environments. Finally, if our model for the role of PAT-12 is correct, both have a critical link to the actin cytoskeleton. As was the case with mutations in the vertebrate kindlin-1 gene, significant phenotypes occur when this linkage is lost or weak. Taken together, there is enough evidence that further study of the C. elegans fibrous organelle will yield information that will be applicable to the vertebrate hemidesmosome.

Questions for Future Study

The differences between our data and that reported by Hetherington et al. regarding the capacity of the different isoforms of PAT-12 to rescue the mutant phenotype suggests that the sequences of the predicted isoforms may not be absolutely correct. To fully determine which isoform(s) are able to rescue, future study should focus on which isoforms are actually transcribed by the organism. To this end, we propose to undergo RT-PCR analysis of the transcripts of pat-12. This could also potentially identify new transcripts that have not been proposed prior to this. Another benefit of this line of study would be that, when worms at different stages are used as the source of the mRNA, one could examine if there is indeed a difference in the isoforms expressed over the course of development. While our data suggests that this is the case, it does have the limitation of
being based on a transgenic rescued animal whose gene number and expression, though proposed to be very similar to wild type, may not be so (that is, these animals may have an increased pat-12 gene copy number, for example). Only by examining real expression at a known point in development can we truly state that expression changes over time in wild type animals.

The work described above allows for only a small of understanding both how the fibrous organelle and the hemidesmosome form and change over time. To begin to tease apart this we will first need to establish that PAT-12 is part of a link between fibrous organelles and the CFBs. Additionally, we will need to identify conclusively the other members of this link beginning with characterizing the ZK849.2 protein. From here, we would like to identify other members of the FO, both Pats and non-Pats. Furthermore, given the changed in FO protein expression over development seen between the different PAT-12 isoforms, we would like to know if the FO present at larval molting stages is composed of the same proteins as that present during embryogenesis. Finally, when we have put this altogether, we will focus on elucidating the complex series of events that allow for FO formation and correct spatial localization. Once we know this, we can use it as a template for understanding the process by which a hemidesmosome forms.

Once the identities and sequences of expressed isoforms have been identified and other members of the FO complex have been identified, two distinct lines of study could take place. The first of these would be examination of the isoforms structure and sequence for similarities with vertebrate proteins as well as differences between isoforms. As described above, previous sequence analysis has failed to produce any vertebrate homologs or identified domains. Any differential splicing could alter these results,
however. Additionally, while the sequences may not be identical, protein structure could be similar enough to suggest overlapping function between PAT-12 and a vertebrate hemidesmosome protein. Protein folding analysis and 3D structure modeling could suggest this as well as giving information about differences in conformation of different isoforms, which could explain their differential expression during development. Finally, by knowing the sequences of expressed transcripts and by seeing what sequences likely form acting sites in the 3D modeled protein, one could hypothesize which protein domains form the key parts of the molecule. This data could guide mutant generation to elucidate which domains are necessary for PAT-12 function.

The other line of experimentation that would now be possible would be those that would identify the temporal organization of the FO, both during initial formation and with changes over the course of development. Specifically, we would determine mutations in which genes lead to mislocalization of PAT-12. Conversely, we could find what proteins are mislocalized in pat-12 mutants, as Hetherington et al. began to do. Once these interactions are known, it would theoretically be possible to determine the structure of the FO in space, especially through the use of electron microscopy as Hetherington et al demonstrated. By using our own binding data as well as the data from large scale Yeast-2-Hybrid screens, it would be possible to identify all FO members and might lead to the discovery of vertebrate homologs. All these lines of experimentation would be possible and would result in a greater understanding of the FO and possibly the vertebrate hemidesmosome thanks to what we now know about PAT-12.
Figure 4.1: Predicted Model of PAT-12 Function. PAT-12 is predicted to link the fibrous organelle to the circumferentially oriented actin fibers through ZK849.2 and VAB-19. Linking these two structures provides additional support especially at time when the cuticle is not present.
Figure 4.2: Schematic Representation of Force Vectors Resulting From Muscle Contractions in Different Areas of Two-fold Embryo. (A) An embryo arrested at the two-fold stage. (B) Contraction of muscles (gray box) in areas of the embryo that remain straight have offsetting forces (black arrows) as seen by the hypodermal cell (red box). (C) At the head and tail, a force vector (blue arrow) is present in the direction of the worm body when the muscle cells contract. This likely causes the muscle cells to detach when PAT-12 is absent. (D) Likewise, at bends in the embryo, the muscle cells are no longer 180 degrees apart from one another and thus a force vector is present, causing muscle detachment described in Hetherington et al.
REFERENCES


