THE ROLE OF THE dim-1 GENE IN MUSCLE MAINTENANCE AND STABILITY IN Caenorhabditis elegans.

by

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**Abstract**

*Unc-112* and *dim-1* are a pair of interacting genes that are required for myofilament lattice assembly and maintenance in the nematode, *Caenorhabditis elegans*. The *unc-112* gene encodes a novel protein localized to attachment structures that are responsible for anchoring the myofilament lattice to the muscle cell membrane and underlying body wall layers. Loss of UNC-112 results in the failure of myofilament lattice assembly and lethality. Animals homozygous for the missense mutation *unc-112 (r367)*, on the other hand, survive to adulthood but are paralyzed and have severely disorganized body wall muscle. Mutations in the *dim-1* gene can suppress the phenotypic defects associated with *unc-112 (r367)*. Animals homozygous for both *dim-1* and *unc-112 (r367)* display wild type movement and have relatively well organized body wall muscle. Animals homozygous for the *dim-1* mutation alone display mildly disorganized muscle, thus the *dim-1* gene is required for maintaining muscle stability.

The *dim-1* gene encodes a 325 amino acid protein that constitutes three immunoglobulin repeats that are most similar to the intracellular muscle proteins, titin and twitchin. Immunofluorescence analysis revealed that DIM-1 is expressed in body wall muscle in a pattern reminiscent of myofilament associated proteins. Preliminary results suggest DIM-1 may associate with actin containing thin filaments. The disorganized muscle phenotype of *dim-1* mutants and the localization of its gene product suggest that DIM-1 maintains the integrity of the myofilament lattice through the stabilization of thin filaments.

Results presented in this thesis suggest that the suppression of *unc-112 (r367)* by *dim-1* is indirect. First, sequence alterations for eight *dim-1* alleles have been identified all of which result in the loss of the *dim-1* gene product. Thus, the absence of DIM-1 results in the suppression of *unc-112 (r367)*. Second, DIM-1 is not required for localization of UNC-112 to attachment structures and third, the DIM-1 protein is localized to myofilaments rather than attachment structures. These results indicate that the genetic interaction between *dim-1* and *unc-112* is not due to a direct interaction between their gene products. Rather, suppression of *unc-112* appears to result from a change in the overall stability of the myofilament lattice caused by the loss of *dim-1*. This change may allow the altered *r367* protein to maintain the integrity of the myofilament lattice.
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<tbody>
<tr>
<td>bp</td>
<td>basepairs</td>
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<tr>
<td>Ca**</td>
<td>calcium</td>
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<tr>
<td>dpy</td>
<td>dumpy</td>
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<tr>
<td>dim</td>
<td>disorganized muscle</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>kb</td>
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<td>ng</td>
<td>nanogram</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pat</td>
<td>paralyzed, arrested elongation at two-fold</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>picomole</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
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<tr>
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<td>μl</td>
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<td>microgram</td>
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<td>uncoordinated</td>
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Chapter 1. INTRODUCTION

1.1 Cytoskeletal networks: The myofilament lattice of muscle.
Cytoskeletal networks are highly ordered arrangements of structural and regulatory proteins that are responsible for changes in cell shape, cell movement and attachment, organelle transport, and cell division (reviewed in Drubin and Hirokawa, 1998). While cytoskeletal structures are extremely diverse in morphology and function, they are built upon a foundation of a small number of highly conserved structural and motor proteins. These include actin and tubulin filaments, which make up the structural framework upon which myosin, kinesin and, dyenin motors interact with to perform energy dependent movement (Drubin, 1998). The diversity within these networks arises from the large number of regulatory and accessory proteins that interact with these main structural components. Through the differential expression and regulation of structural accessories, the cytoskeleton becomes an intricate and dynamic system capable of performing highly specific functions. How does the cell regulate the assembly of cytoskeletal networks to perform their specific functions at the proper time during the life of a cell? What are the structural and regulatory components involved? How do they interact with one another? These are central questions in cell biology that have been approached by genetic and biochemical dissection of specific cytoskeletal structures such as the spindle apparatus of a mitotic cell or the polarized bud growth of a dividing yeast cell (Salmon and Way, 1999).
The contractile apparatus of muscle is one of the most familiar and highly ordered cytoskeletal structures responsible for movement in higher eukaryotes. It is a stable structure that must maintain contractions required for tissue and organ movements. At the same time, it is a dynamic structure that must grow and alter its morphology to accompany the changing motility requirements of a developing organism. The structure of muscle is well described and highly conserved among vertebrates and invertebrates. Thus the myofilament lattice of muscle has become a model for studying the assembly of cytoskeletal networks. A main focus in our lab is to understand the mechanisms that govern the assembly and maintenance of the muscle myofilament lattice. To approach this goal, we use body wall muscle of the nematode Caenorhabditis elegans as a model.

Striated muscle is characterized by a highly ordered array of repeating structures called sarcomeres, the contractile units of myofibrils. A sarcomere consists of interdigitating myosin thick filaments and actin thin filaments, which slide past each other producing contractile force. The sarcomere is delineated on either end by Z-disks, which are sites of attachment for thin filaments. The M-line (midline) of the sarcomere is the location of thick filament attachment. The striated appearance of muscle results from regions containing overlapping thick and thin filaments (A-bands), alternating with regions containing non-overlapping thin filaments (I-bands) and non-overlapping thick filaments (H-zone) (see Figure 3).

In order for sarcomeres to generate force, contractile proteins must be linked to the muscle cell membrane, extracellular matrix (ECM) and skeletal structures. This is accomplished through special adhesion sites between myofibrils and extracellular components through the plasma membrane. The primary sites of attachment in vertebrate skeletal muscle are located at the ends of myofibrils and contain talin, vinculin, alpha-actinin, fibronectin, tenascin, and integrin (reviewed in Epstein, 1991). The anchorage sites linking the myofilament lattice and the exoskeleton in nematodes are primarily lateral and occur at intervals along the entire length of the cell coincident with the sites of attachment for actin (Z-disk) and myosin filaments (M-line). This arrangement is reminiscent of anchorages found in vertebrate smooth and cardiac muscle cells (Francis and Waterston, 1985, Imanaka-Yoshida et al., 1999). Invertebrate adhesion sites contain
integrin, vinculin, alpha-actinin, talin, and other membrane/ECM associated proteins (Gettner et al., 1995, Barstead and Waterston, 1985, Moulder et al., 1996).

Perhaps the most fascinating and perplexing aspect of the contractile apparatus is how it is assembled from its many constituents into an intricately defined myofilament lattice. The precise organization of thin and thick filament lattices in sarcomeres cannot be explained by the self-assembly properties of actin and myosin alone (reviewed in Obinata, 1993). A complex cytoskeletal framework is involved whose number of constituents has only recently been appreciated. While many structural components of this framework have been identified, the mechanisms governing their organization into a complex lattice remain unclear. These structural components can roughly be placed into two classes, those that are associated with the muscle cell membrane (or sarcolemma) and filament attachment structures, and those that are associated with the filaments themselves. The importance of membrane associated components during the early phases of sarcomere assembly has been demonstrated in many systems (Williams et al., 1994, Hresko et al., 1994, Volk et al., 1990, Lu et al., 1992). The initial stages of muscle assembly occur near the membrane in primary cultures of muscle cells, and attachment components are localized to the membrane during early myofibril assembly (Lu et al., 1992). Nematodes with null mutations in genes encoding membrane associated proteins such as vinculin, beta-integrin, or the ECM protein, perlecan, fail to assemble a myofilament lattice (Barstead and Waterston, 1991, Rogalski et al., 1993, Williams et al., 1994). Beta-integrin is also required for proper muscle assembly in Drosophila (Volk et al., 1990). Therefore, components of membrane/ECM linkages not only serve as anchorages for transmission of contractile force, but also function in directing the early stages of contractile filament assembly.

Muscle components that associate with myofilaments are also important for lattice assembly. The presumptive molecular rulers titin and nebulin localize to the A-bands and I-bands in vertebrate muscle and are thought to regulate thick and thin filament length respectively (Trinick, 1994). The giant UNC-89 protein of the immunoglobulin superfamily localizes to A-bands and is required for thick filament maintenance in C. elegans (Benian et al., 1996). Muscle proteins continue to be identified and the roles they play in building and/or regulating the sarcomere are just beginning to be understood.
Further work focusing on the interactions of structural and regulatory elements will be crucial to understanding how complex structural protein networks are assembled.

Another important element in building a contractile apparatus is its structural maintenance. Once muscle is assembled, many interactions are required to maintain the stability of the filament lattice. These interactions must regulate the muscle structure during contraction and allow for growth. It is clear that some of the essential components for initial sarcomere assembly also play a role in maintaining muscle structure during contraction and growth. Additionally, there are muscle components whose primary roles appear to be involved in stabilizing and maintaining assembled muscle (reviewed in Moerman and Fire, 1997).

I am interested in the components that regulate the assembly, growth and maintenance of the myofilament lattice in striated muscle. Understanding how these components interact with each other and contractile filaments is central to understanding the assembly and growth processes that build muscle. The current approaches used to dissect muscle components and address their roles in muscle assembly include biochemical and methods based on protein interactions in vertebrate cultured muscle cells (Lu et al., 1992) and genetic approaches to identify muscle specific genes using model invertebrates such as Drosophila and C. elegans.

The nematode Caenorhabditis elegans is ideally-suited for the study of muscle assembly. Using genetic, molecular, and biochemical approaches, over 80 genes affecting muscle structure and function have been identified in C. elegans (reviewed in Moerman and Fire, 1997) and approximately 40 antibodies have been generated that recognize muscle associated proteins (Francis and Waterston, 1985 and 1991, reviewed in Waterston, 1988). The Genome Sequencing Consortium has recently completed sequencing the entire C. elegans genome (C. elegans Sequencing Consortium, 1999) and a complete physical map of overlapping cosmids and YAC’s is available. An extensive genetic map of known C. elegans genes has also been compiled. These resources are invaluable for the identification and analysis of genes encoding muscle components in C. elegans. Using this set of tools, we aim to characterize the genes and their protein products that are required for muscle assembly and maintenance in C. elegans with the
hopes of contributing to the understanding of muscle structure and function in all organisms.

1.2 Anatomical description of muscle in *C. elegans*

*C. elegans* adult body wall muscle is composed of 95 muscle cells that are arranged in four quadrants, two dorsal, and two ventral, that run longitudinally along the length of the worm (Figure 1). The cells do not fuse to form a multinucleate myotube as in vertebrate skeletal muscle, rather individual mononucleate cells within a quadrant adhere tightly to one another in an overlapping fashion. The muscle cells are polarized such that the myofilament lattice is located adjacent to the cell membrane associated with the underlying ECM (basement membrane), hypodermis, and cuticle (review in Waterston, 1988).

Thick and thin filaments are oriented parallel to the longitudinal axis of the worm, but adjacent sarcomeres are staggered relative to one another rather than aligned as in vertebrate muscle. This arrangement results in A-bands and I-bands that are oriented at a 6-degree angle to the longitudinal axis of the worm and to the filaments themselves (Figure 4). Thus, the overall lattice appears obliquely striated when viewed under polarized light (reviewed in Waterston, 1988).

The principal component of thick filaments is myosin, although thick filaments in nematodes also contain paramyosin (Epstein et al., 1974; Waterston et al., 1974). Thin filaments contain actin, tropomyosin, and troponins C, I, and T (Epstein et al., 1974; Waterston et al., 1974; Nakae and Obinata, 1993; Kagawa et al., 1995). Thick and thin filaments are anchored to the membrane and ECM through M-lines and dense bodies (the analog of vertebrate Z-disk), respectively (Figure 2; Francis and Waterston, 1985). Dense bodies are similar to focal adhesion sites in mammalian non-muscle cells and contain integrin, vinculin, UNC-97/PINCH, alpha-actinin, UNC-112, ILK, and talin (Figure 3; Barstead and Waterston, 1991, Moulder et al., 1996, Rogalski, unpublished results, Hobert et al., 1999, Williams, personal communication). The composition of the M-line is less defined, but includes integrin (Francis and Waterston, 1985; Gettner et al., 1995), UNC-112 (Rogalski, unpublished results), PINCH (Hobert et al., 1999), ILK (Williams, personal communication), and the *unc-89* gene product (Benian et al., 1996).
Figure 1. Schematic view of *C. elegans* muscle organization.

A. Longitudinal view of the worm. *C. elegans* spindle shaped body wall muscle cells form a double row and run longitudinally the entire length of the worm. B. Polarized light view of a muscle cell. C. The body wall muscle is arranged in four quadrants that lies just beneath the hypodermis as shown in this cross section. D. An individual muscle cell. E. The structural components of sarcomeres (actin and myosin based contractile units) and their anchorage to the outer body wall layers is shown. The anchorage units (or dense bodies) are similar to focal adhesions of vertebrate cells and contain integrin, vinculin, alpha-actinin, talin, and other proteins.
Figure 2. Schematic of a single *C. elegans* sarcomere.

A sarcomere consists of interdigitating myosin thick filaments and actin thin filaments. Thin filaments are attached to the dense body, and thick filaments are attached to the M-line. The M-lines and dense bodies link the sarcomere to the underlying ECM, hypodermis, and cuticle. Proteins that are important for these linkages are labeled according to their spatial distribution within the sarcomere. Schematics of magnified actin and myosin filaments are depicted above.
Figure 3. Components of the *C. elegans* body dense body.

The *C. elegans* dense body is similar to focal adhesions of vertebrate non-muscle cells. The schematic depicts the relationship among components of the dense body as understood to date. Perlecan may directly interact with alpha and beta-integrin to nucleate the formation of the dense body (Hresko *et al.*, 1994, Rogalski *et al.*, 1995). The nematode homologues of ILK and FAK have been identified, but the localization of FAK is not yet known (B. Williams, personal communication, Barstead, personal communication). Vertebrate studies indicate that PINCH interacts directly with ILK (Tu *et al.*, 1999). Vinculin, talin, and alpha-actinin have all been shown to interact with actin in vertebrate systems (Burridge, 1996) (From Moerman, unpublished).
perlecan (unc-52)

DIM-1 ⇔ UNC-112 ⇔

?  alpha and beta integrin (pat-2, pat-3)

ILK (pat-4) ↩

FAK ?

talin (unc-35)

vinculin (deb-1)

alpha-actinin

actin filament (act-123, pat-10, lev-11 etc)
Figure 4. Structure of body wall musculature as viewed by light microscopy.

(a) and (b). Illustration of the relationship of the contractile units to the pattern of muscle organization as seen through the light microscope. (c). High magnification polarized light micrographs of adult muscle. (d). Low magnification polarized light micrograph of adult muscle showing two muscle quadrants. **Large arrow:** M-line. **Open arrow:** I-band. **Small arrow:** dense body. **Curved arrow:** cell margin (From Waterston, 1988).
The transmembrane receptor, integrin, is found at the base of dense bodies and M-lines, and anchors these structures to the basement membrane (Gettner et al., 1995). This attachment is mediated by the basement membrane protein, perlecan (Rogalski et al., 1995). Muscle cells are anchored to the cuticular exoskeleton through hemidesmosome structures of the hypodermis (Figure 3; Francis and Waterston, 1991). This interaction allows for the mechanical force of contraction to be transduced into movement.

1.3 Myofilament lattice assembly in *C. elegans*

As sarcomeres assemble during embryogenesis, muscle components accumulate at the plasma membrane (Hresko et al., 1994). Evidence suggests that the membrane associated molecules such as integrin, perlecan, UNC-112, and vinculin may dictate the spatial assemblage of more internal components (Hresko et al., 1994, Willaims et al., 1994, Rogalski et al., 1993 and unpublished results, Barstead and Waterston, 1991). One model proposes that integrin and perlecan may form nucleating centers for assembly and several other components assemble independently onto these centers (Hresko et al., 1994, Rogalski et al., 1995, Mullen et al., 1999, reviewed in Moerman and Fire, 1997). This suggestion is particularly attractive since integrin has been shown to be involved in cell adhesion, signal transduction, and cytoskeletal organization in vertebrate systems (Burridge, 1996).

Once the embryonic lattice is assembled, contraction begins and the embryos elongate prior to hatching as L1 larvae. The animals pass through three subsequent larval stages before reaching maturity. During this interval, muscle lattices grow many times their size at hatching. The number of A-bands per cell increases from two in embryos to ten in adults. The distance between M-lines and dense bodies also increases during growth (reviewed in Moerman and Fire, 1997). That is, not only do the sarcomeres increase in number during growth, but the size of individual sarcomeres also increases by the integration of additional filaments, and by the increase in length of existing filaments. Therefore, this highly ordered lattice must be very flexible to allow for integration of new components into an established structure while maintaining the overall organization of the lattice during the growth process.
1.4. Mutations affecting muscle in *C. elegans*.

Over 80 muscle-affecting genes have been described in *C. elegans* (reviewed in Waterston, 1988 and Moerman and Fire, 1997, see Appendix B, and see web page http://www.zoology.ubc.ca/labs/moerman/muscle_affecting_genes_.html). Mutations affecting muscle can be classified into a few categories. Null mutations in genes encoding muscle assembly proteins such as beta-integrin, perlecan, myosin heavy chain A, and vinculin fall into the PAT (paralyzed, arrested at two-fold) class of mutations (Williams et al., 1994, Rogalski et al., 1993, Waterston 1989, Barstead and Waterston, 1991). This class of muscle mutations affects the initial assembly process, thus animals fail to build a myofilament lattice, do not undergo elongation, and arrest early in embryogenesis.

Mutations falling into the UNC class primarily affect the stability of muscle causing the structural integrity of muscle to be compromised during growth and contraction. Initial assembly proceeds normally in these mutants, but myofilaments become progressively disorganized throughout the late stages of development (Moerman and Fire, 1997). In extreme cases, the animals are paralyzed as adults while in milder cases they may merely move sluggishly or in an uncoordinated fashion (*unc*). Certain missense mutations in actin and myosin as well as mutations in the immunoglobulin superfamily proteins, *unc-22* and *unc-89*, fall into this *unc* class (reviewed in Moerman and Fire, 1997, Benian et al. 1989, 1996, Waterston et al., 1984). The other classes of muscle-affecting genes include the MUA (muscle attachment, J. Plenefisch, personal communication) and the MUP (muscle positioning, Hedgecock et al., 1987; Goh and Bogaert, 1991) mutants, both of which affect muscle attachment and lead to lethality.

Some membrane-associated proteins that are required for initial muscle assembly in embryos have additional functions in the maintenance and growth of body wall muscle. The *unc-52* gene encodes the basement membrane proteoglycan, perlecan, which is localized to attachment structures in muscle (Rogalski et al., 1993). Tissue and temporal specific isoforms of perlecan are expressed in muscle, where mutations that affect a certain subset of perlecan isoforms lead to late larval defects such as disorganization and detachment of muscle (Rogalski et al, 1995). Clearly these isoforms are dispensable for embryonic assembly, but are required for growth and stability of the myofilament lattice. Another example is the *unc-112* gene, which also falls into the *pat*
class of muscle-affecting genes. Animals carrying \textit{unc-112} null mutations fail to assemble a myofilament lattice and arrest as embryos (Williams et al., 1994). In contrast, \textit{unc-112} viable mutations lead to disruption of muscle organization in late larval mutations (Gilbert, 1997). Thus, the \textit{unc-112} gene product is involved in maintaining the structural integrity of muscle as well as playing a role in the initial assembly.

Further exploration into \textit{unc-112} function has revealed an interacting gene, \textit{dim-1}, that is also required for the maintenance and stability of muscle (Gilbert, 1997). The nature of the interaction between these two genes and the role of the \textit{dim-1} gene in muscle stability is unclear. The remainder of this thesis will focus on these two genes, with special consideration to \textit{dim-1} and its role in myofilament stability and its interaction with \textit{unc-112}.

1.5. \textit{unc-112} encodes a novel protein that is localized to attachment structures in body wall muscle.

The \textit{unc-112} gene encodes a 720 amino acid protein that is expressed at the M-lines and dense bodies of body wall muscle (Rogalski, unpublished results). The UNC-112 protein shares significant homology to the human MIG-2 (mitogen-inducible gene) whose function remains unknown. UNC-112 and MIG-2 share a short region of homology to members of the FERM superfamily of proteins including talin, Band 4.1 and ezrin. This region may be important for anchoring these proteins to the plasma membrane (Chishti, et al., 1998). UNC-112 is localized to the dense bodies, M-lines, and cell margins of body wall muscle and colocalizes with integrin at the membrane-associated base of these attachment structures. Both mutant analysis and localization studies suggest that UNC-112 is involved in organizing the spatial distribution of dense body and M-line components upon which myofilaments are added and anchored by. The additional role for UNC-112 in maintaining muscle integrity during growth is suggested by the phenotype of the \textit{unc-112} missense allele, \textit{(r367)}.

1.6. The \textit{dim-1} mutation results in suppression of the \textit{unc-112} \textit{(r367)} phenotype.

Prior to the phenotypic characterization of \textit{unc-112} null mutants, and to the cloning of \textit{unc-112}, the \textit{r367} allele was used to explore the roles of the \textit{unc-112} gene in assembly
and maintenance of muscle. To identify genes that interact with \textit{unc-112}, a screen for intergenic suppressors was performed on \textit{unc-112 (r367)} viable mutants. Several extragenic suppressors were identified by isolating well-moving animals from the progeny of mutagenized \textit{unc-112 (r367)} homozygotes. Of the suppressors characterized, all are alleles of the \textit{dim-1} (disorganized muscle) locus, positioned on the X-chromosome (Gilbert, 1997). Worms carrying both the \textit{unc-112 (r367)} and \textit{dim-1} mutations exhibit nearly wild type movement and have relatively well organized body wall musculature (Figure 5). However, mutations in \textit{dim-1} cannot suppress the lethal phenotype caused by null mutations in \textit{unc-112}. The \textit{dim-1} mutations are thought to be null and can suppress the \textit{unc-112 (r367)} phenotype when either heterozygous (good suppression) or homozygous (better suppression). Thus, reduction or loss of the \textit{dim-1} gene product results in the suppression of \textit{unc-112 (r367)} phenotype. Animals carrying the \textit{dim-1} mutation alone are wild type in size and movement, but the body wall muscle appears disorganized under the polarized light microscope. Many sarcomeres display a characteristic “chevron” phenotype when A-bands are often seem intersecting each other at an acute angle rather than lying parallel as in wild type. The overall muscle structure is more fragile than wild type and the myofilament lattice is easily disrupted when manipulated (Figure 5) (Gilbert, 1997). Hence, \textit{dim-1} is required for the stability of the myofilament lattice.

The \textit{dim-1} gene was initially mapped between \textit{dpy-6} and \textit{dpy-7} on LGX. Transformation rescue placed the gene on a single cosmid, C18A11, narrowing down the \textit{dim-1} locus to five possible open reading frames (Moerman and Rogalski, unpublished results). The presence of cDNA clones in the Y. Kohara database corresponding to the predicted ORF C18A11.7 made this a likely candidate for \textit{dim-1}. While the Kohara database does not yet completely represent all the transcribed genes in \textit{C. elegans}, we made this assumption on the basis that cDNAs encoding muscle proteins are usually highly represented in libraries owing to the abundance of muscle proteins in cells. Putative deletion alleles of \textit{dim-1} were generated which aided in the cloning of the \textit{dim-1} locus, and the sequence alterations in three of these alleles were determined. All three alleles contain deletions within the candidate region, thus the \textit{dim-1} gene corresponds to the ORF, C18A11.7. Two of these deficiencies, \textit{ra209} and \textit{ra114}, remove the entire
Figure 5. Polarized light images of body wall muscle. A. Wild type. Note the highly organized structure; A-bands and I-bands alternate keeping entirely parallel.

B. unc-112 (r367). Muscle is severely disrupted. C. dim-1 mutant showing a cheveroned muscle cell. Note the intersection of filaments coming in at an acute angle.

D. unc-112 (r367); dim-1. Muscle is still disorganized, but substantially better than unc-112 mutants alone.
ORF while *ra111* removes 183 bp beginning in exon 2 and extending into the adjacent intron (Figure 6) (Rogalski and Devenport, unpublished data).

Interestingly, one cDNA found in the Kohara database contains coding sequence extending into the upstream open reading frame, C18A11.8, suggesting that these two ORF's, C18A11.7 and C18A11.8, may actually constitute one gene containing a large (3.5 kb) intron rather than two separate gene's as Gene Finder predicted. We sequenced this clone and found that its 5' end begins near the middle of exon 5 of C18A11.8 and extends downstream of the stop codon in exon 6 (12?) of C18A11.7 (Figure 6) (Rogalski and Devenport, unpublished data). All predicted splicing sites were conserved suggesting that this cDNA is a product of an authentic mRNA rather than an artifact produced during library construction. The first five predicted exons in C18A11.8 were amplified by PCR off another *C. elegans* cDNA library (provided by Bob Barstead) confirming that all twelve exons are transcribed in the worm. These two pieces of evidence strengthened the suggestion that these ORF's actually comprise one gene rather than two.

1.7. Does the *dim-1* gene encode a novel 640 amino acid protein required for myofilament lattice stability?
The *dim-1* gene has been shown to interact genetically with the *unc-112 (r367)* mutation and play a role in myofilament stability. The *dim-1* gene has been cloned and corresponds to the ORF C18A11.7 and perhaps C18A11.8 as well. If *dim-1* does correspond to both open reading frames, it is predicted to encode a 640 amino acid protein that is presumably involved in stabilizing and maintaining the integrity of the myofilament lattice. The first 315 amino acids (corresponding to C18A11.8) are not similar to any known protein whereas the remaining 325 amino acids (corresponding to C18A11.7) constitute three immunoglobulin repeats of the intracellular type most similar to those found in the muscle proteins UNC-22/twitchin and titin (Figure 6).

How precisely does *dim-1* affect the stability of the myofilament lattice? How do mutations in *dim-1* suppress the muscle phenotype associated with *unc-112 (r367)*? The primary focus of this thesis is to address these two fundamental questions. I approached
Figure 6. Schematic of the C18A11.7 and C18A11.8 open reading frames.

The two open reading frames are separated by 3.5 kb of intergenic space. C18A11.8 encodes a novel sequence of 315 amino acids, and C18A11.7 encodes 325 amino acids that make up three immunoglobulin repeats. The cDNA clone, yk399b7 contains sequence from both of these ORF suggesting that these two genes are actually one. The cDNA clone contains coding sequence spanning the region shown below.
this problem by analyzing the molecular lesions associated with various $dim-1$ alleles, discovering the expression and localization of the $dim-1$ gene product, and addressing the requirement for DIM-1 in UNC-112 localization.

The experiments performed in this thesis were designed with the notion that the $dim-1$ gene encodes a 640 amino acid protein spanning approximately 8 KB of genomic sequence. However, the results of many of these experiments suggest this may not be the case, thus another focus has been added to this thesis: to determine whether $dim-1$ encodes a 640 amino acid protein corresponding to both of the open reading frames, C18A11.7 and C18A11.8.
Chapter 2. MATERIALS AND METHODS

2.1. Nematode Strains and culture conditions

Nematode strains were grown on NGM plates streaked with *Escherichia coli* (OP50 strain) as described in Brenner, 1974). All strains were maintained at 20° C, unless otherwise noted. Nematode strains were either generated in the Moerman lab or obtained from the *Caenorhabditis* Genetics center (CGC).

*C. elegans* strains used in this work include the wild type strain N2; CB444, *dim-1*(ra102); DM2, *dim-1*(ra214); DM1210, *dim-1*(ra215); DM1213, *dim-1*(ra204); DM1244, *dim-1*(ra203); DM1247, *dim-1*(ra219); DM1221, *unc-112:*gfp(ex16); DM7016, *dim-1*(ra102); *unc-112:*gfp(ex16); DM2706.

2.2. Genetics

To introduce the *unc-112:*gfp(ex16) extrachromosomal array into *dim-1*(ra102) mutants, N2 males were crossed to *dim-1*(ra102) hermaphrodites. The outcross *dim-1* hemizygous male progeny were then crossed to *unc-112:*gfp(ex16) carrying hermaphrodites. Outcross progeny were selected by scoring for the dominant “roller” phenotype that results from the presence of the prf4 plasmid that is coexpressed with the *unc-
112::gfp(ex16) chromosomal array. These outcross progeny were singly picked to new plates and allowed to self-reproduce. "Rollers" segregating only dim-1 progeny were selected and used for further morphological analysis.

2.3. Fusion protein construction and expression

The plasmid, DM#709, was used for fusion protein expression in the DH5α strain of bacteria. DM#709 was constructed by subcloning a fragment of the dim-1 coding sequence corresponding to exons 5 and 6 of ORF C18A11.8 and exons 1 through 4 of ORF C18A11.7 into the GST expression vector, pGEX-1. The fragment of dim-1 coding sequence was obtained from the plasmid DM# 203, a pBLUESCRIPT vector with a dim-1 cDNA insert constructed by Teresa Rogalski. DM#203 was cut with the restriction endonucleases BamH1 and EcoR1. The 550 bp fragment of interest was cut from a 1% agarose gel and purified using a Qiagen Gel Extraction kit and subcloned into the pGEX-1 vector.

To analyze the expression of the GST-fusion protein, cell lysates were prepared from cultures after induction with IPTG. 0.1 ml of overnight culture was added to 25 ml fresh 2xYT media and then grown for 90 min. IPTG (Boehringer Mannheim) was added to 1mM and the culture was grown another 90 min. Cells were then pelleted by centrifugation and resuspended in 1X Laemmli sample buffer (50mM Tris-Cl (pH 6.8), 100mM dithiothreitol, 10% glycerol, 2% SDS, and 0.1% bromophenol blue), and boiled for 5 min. SDS-PAGE and coomassie staining were then used to analyze 5μl of each sample.

Large-scale preparation and purification of the GST fusion protein was performed as described by Smith and Johnson (1988) with some modifications. Briefly, 25 ml overnight cultures were grown at 37° C in 2XYT media supplemented with 100ug/ml ampicillin. 10 ml of overnight culture was added to 250 ml of fresh medium and grown for 2 - 3 hours at 37° with shaking. IPTG was added to a final concentration of 1 mM, and cultures were grown an additional 2 - 4 hours. Cells were pelleted by centrifugation at 10,000 RPM and resuspended in MT-PBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄) supplemented with PMSF and EDTA to a final volume of 9 ml. Cells were lysed by gentle sonication (6 x 30 sec. with chilling on ice between bursts). 1 ml of 10%
Triton X-100 (in MT-PBS) was added and the suspension incubated for 5 min. at 4°. Insoluble debris was pelleted by centrifugation at 10,000 RPM and the supernatant transferred to a 15 ml conical centrifuge tube containing 2 - 3 ml pre-swollen glutathione-agarose beads (Sigma Chemical Company). The tube was rotated gently for 5 min. at room temperature. Beads were washed 5x in MT-PBS + 1% Triton X-100, followed by 2x in MT-PBS without detergent. Bound fusion protein was eluted 3x at room temperature with 1 volume of elution buffer (50 mM Tris-CL, 10 mM glutathione) and stored at -20°.

2.4. Generation of polyclonal antisera
To generate polyclonal antisera, New Zealand White rabbits were injected subcutaneously with purified fusion protein emulsified in Freund's complete adjuvant (approximately 0.2 mg protein/rabbit). Two rabbits were used, and serum from one of these was chosen for further study. Rabbits were boosted at 4-8 week intervals with fusion protein emulsified in Freund's incomplete adjuvant (approximately 0.25 mg protein/rabbit) and blood samples were taken 10 to 12 days post injection. Immune response was monitored by Western blotting of purified fusion proteins and immunofluorescence staining. In all cases, antisera from these replicates gave similar results in Western blotting and immunofluorescence experiments.

2.5. Western blotting
For Western blots on worm protein extracts, mixed staged worms were washed off plates with dH20 and pelleted by centrifugation. The worms were resuspended in 2X Laemmli sample buffer at a 1:1 ratio, worms: sample buffer. The samples were boiled for 5 min, sonicated for 30 sec., and boiled again for 5 min. 10 μl of each sample of worm lysates were loaded onto 10% polyacrylamide gels. Proteins were resolved by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham) for 30- 45 min. at 10 V in a Trans-Blot SD Electrophoretic Transfer Cell (BioRad). Blots were blocked for 1-3 hours in 5% milk powder-TBS-T (TBS-T: 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20) and incubated with primary antibodies (see below) in 1% milk powder-TBS-T overnight at room temperature. After washing in TBS-T, blots were incubated with
horseradish peroxidase-labelled secondary antibodies (see below) in 1% milk powder-TBS-T for approximately 45 min. at room temperature. After washing in TBS-T and in TBS, blots were incubated for 1 min. in ECL detection reagents (Amersham) and exposed to film (Kodak X-OMAT).

For Western blotting, rabbit polyclonal serum was diluted 1:5000 to 1:10,000, and horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibodies were diluted 1:10,000.

2.6. Immunofluorescence Staining

Mixed stage worms were prepared for immunofluorescence staining following the freeze crack method adapted from Albertson (1984). Worms were washed off a small NGM plate with water and pelleted by gentle centrifugation (1500 RPM). The worms were resuspended in 4% sucrose, 1mM EDTA, and pelleted again. Most of the sucrose solution was removed leaving a ~250 μl solution of suspended worms. 20-25 μl of worm suspension were pipetted onto a polylysine-coated slide and a 24 x 50 (thickness 2) coverslip placed on top. The slides were then placed on a aluminum block (pre-cooled to -80°) and stored at -80° until needed. For staining, the coverslips were flipped off and the slides placed in a coplin jar containing ~20° acetone and fixed for 4 min. The worms were then rehydrated through a graded acetone series (4°). The slides were washed in TBS-T and placed in humidified chambers. 50-100 μl primary antibody (in 1% BSA, TBS-T) were pipetted onto the worms and allowed to sit overnight at room temp. The worms were then washed extensively in TBS-T, placed again in humidifying chambers and 50-100 μl secondary antibodies were pipetted onto the slides. The worms were incubated for 3 hours in secondary antibodies in the dark at room temperature and then washed in TBS-T. 25 μl of mounting media was then pipetted onto the worms and a 24 x 50 coverslip placed on top.

Primary antisera were diluted in 1% BSA, TBS-T to the following concentrations: DD1: 1:100, MH24: 1:250, MH35: 1:250, DM5.6: 1:100, DM5.8: 1:100. Secondary antibodies and FITC labeled phalloidin were diluted to 1:1000.
2.7. Microscopy

Immunofluorescence images were collected on a Zeiss Axiophot microscope equipped with fluorescence optics. Images were collected with a DAGE-MTI CCD 100 camera and viewed in Scion Image 1.60a on a Power Macintosh 7500/100. Images were imported into Photoshop, with RGB color in some cases, and cropped to the desired size and resolution for presentation.

Confocal images (Figure 14) were collected using Bioradiance Plus (BioRad) confocal laser apparatus with a Zeiss inverted microscope. Data was collected using Laser Sharp software. Z-series consisted of 400 X 400 pixel images taken at 0.2 micron intervals for the depth of a single muscle quadrant. Images were analyzed using Scion Image 1.60a and were important into Adobe Photoshop for presentation.

2.8. Sequencing of dim-1 alleles

Six dim-1 alleles, ra102, ra203, ra204, ra214, ra215, and ra219 were sequenced using Big Dye Terminator Automated Sequencing. The two putative dim-1 open reading frames were amplified by PCR using DNA from whole worm lysates of each dim-1 allele as templates. Three adult worms were suspended in 3 μl worm lysis buffer (50 mM KCl, 10mM Tris (pH 8.3), 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 60 ug/ml proteinase K) and heated to 95° for 45 min. For PCR amplification, 1 μl each forward and reverse primers (25 pmol/μl), and 20 μl master mix containing 2.5 μl 10x PCR buffer, 2 μl dNTP’s, 1.25 μl MgCl₂, 14.75 μl dH₂O, 0.5 μl Taq polymerase (Gibco BRL) were added to each tube. The ~2 kb products were amplified for 30 cycles consisting of 30 sec at 95°, 60 sec at 57°, and 2 min at 72°. The PCR products were then purified using a PCR purification kit (Qiagen) and 90 ng of each sample (30 ng/μl) were sent with the appropriate sequencing primer (3.2 pmol/μl) to the NAPS unit where sequencing reactions were performed. The NAPS unit uses AmpliTaq FS Dye Deoxy Terminator Cycle Sequencing chemistry (Applied Biosystems) with the following conditions. 90 ng template DNA (30-90 ng/ul), 3.2 pmol sequencing primer, and 8 μl terminator premix are combined and amplified for 25 cycles consisting of 30 seconds at 96°, 15 seconds at 50°, and 4 minutes at 60° in a Perkin-Elmer Model 480 thermal cycler. Sequencing was
performed on a 373 Stretch Sequencer (Applied Biosystems). Sequences were compared to that of the N2 Bristol strain genomic sequence available from the Sanger center. In each case where a sequence change was found, a new PCR product was amplified and sequenced to confirm the change is a true mutation and not a PCR induced alteration.

2.9. Reverse transcription PCR.
C. elegans RNA was generously provided by E. Mathews. For reverse transcription, 1 μl (0.5 - 1 μg) of total RNA was combined with 1 μl reverse primer (25 pmol/μl), incubated at 70° for 10 min. and chilled on ice. 8 μl of a master mix containing 1 μl 10x PCR buffer (Gibco BRL), 1 μl 10 mM dNTPs (2.5 mM each dNTP), 1 μl 100 mM DTT, 0.5 μl 50 mM MgCl₂, 0.5 μl RNasin, 0.5 μl Superscript II RT (Gibco BRL) and 3.5 μl dH₂O per reaction, was added to each tube. Tubes were incubated at 42° for 60 min. and at 95° for 15 min. For PCR amplification, 1 μl of forward primer (25 pmol/μl) and 14 μl of a master mix containing 1.5 μl 10x PCR buffer, 1 μl 10 M dNTPs, 0.5 μl Taq DNA polymerase (Gibco BRL) and 11 μl dH₂O per reaction, were added to each tube. Reverse transcription products were amplified for 30 cycles consisting of 30 sec at 95°, 60 sec at 50 - 55°, and 90 sec at 72°.
Chapter 3. RESULTS

3.1. C18A11.7 is not trans-spliced to SL1 or SL2.
Gene Finder predicted C18A11.7 and C18A11.8 as two separate genes. One cDNA was found that suggested these two open reading frames may actually comprise a single gene. If these two ORF's are two independently transcribed genes, their corresponding mRNA transcripts should reflect this.

Approximately 70% of C. elegans transcripts are trans-spliced to a short splice leader (Spieth et al., 1993). The most common of these is the SL1 splice leader, which is trans-spliced to the 5' end of single genes (Conrad et al., 1991). Because Gene Finder can often make mistakes predicting the 5' ends of genes and cDNAs are often incomplete representations of gene transcripts, detection of the SL1 splice leader in cDNAs has become a useful means of determining the 5' end of gene. To determine if the 5' end of the C18A11.7 open reading frame is trans-spliced to SL1, and thus an individual gene, RT-PCR was performed on RNA from mixed stage worms using an SL1 forward primer and C18A11.7 specific reverse primer. Control cDNA fragments were amplified with internal primers to the 5' and 3' ends of the predicted coding sequence, but no fragment was amplified when the SL1 primer was used. Thus, C18A11.7 transcripts are not trans-spliced to SL1. However, a similar experiment was performed with the C18A11.8 ORF,
which gave the same result. This time the ORF was amplified by PCR using a randomly primed cDNA library (λAct RB2, donated by Bob Barstead) as DNA template. Again control fragments were amplified using internal primers, but nothing was amplified when the SL1 primer was used. It seems that transcripts from both ORF’s are not trans-spliced to SL1. Unfortunately, only a positive result from this experiment would be conclusive as to what the 5’ ends of these ORF’s are since only 70% of C. elegans transcripts are trans-spliced.

Perhaps C18A11.7 and C18A11.8 are part of an operon and are co-transcribed. The cDNA, yk399b7, may represent a portion of a polycistronic transcript. Polycistronic transcripts from C. elegans operons are also trans-spliced. The first gene in an operon is trans-spliced to SL1 while downstream genes are trans-spliced to either SL1 or SL2 (T. Blumenthal, personal communication). If C18A11.7 is the downstream gene in an operon, it’s mRNA is predicted to become trans-spliced to SL2. Again, RT-PCR was performed using an SL2 forward primer and internal C18A11.7 reverse primer. Control fragments of the appropriate size amplified with internal primers while SL2 primed fragments did not (data not shown). Thus C18A11.7 is not trans-spliced to SL2, and is not likely to be polycistronic with C18A11.8.

3.2. DD1, an antiserum against DIM-1, recognizes a 35 KD protein.

To address when and where the dim-1 gene product is expressed and localized, a polyclonal antiserum against the dim-1 protein was generated. Details of antiserum generation are outlined in Materials and Methods. Briefly, a GST fusion protein was generated containing sequence from exons 5 and 6 of ORF, C18A11.8 and exons 1 through 4 of ORF, C18A11.7. Anticipating that we may generate a serum that cross-reacts with other worm proteins that contain immunoglobulin motifs, coding sequence from the novel portion of the putative dim-1 coding sequence was included in the fusion protein. The fusion protein was expressed in E. coli, purified, and injected into New Zealand White rabbits. Boosts were performed at 30 day intervals and collected sera were analyzed for reactivity to DIM-1 by Western blotting and immunofluorescence staining. One serum, DD1, was chosen for further study.
If the *dim-1* protein is encoded by both ORF's, the predicted protein size is 71 KD. DD1 (GST depleted sera) detects an approximately 35 KD protein on wild type N2 lysates which is the expected size of either ORF C18A11.8 (36 KD) or C18A11.7 (35 KD), but not both (Figure 7). Protein extracts from *dim-1* deletion mutants were prepared and analyzed by western blotting to test if the observed reactivity is specific to the *dim-1* gene product. *dim-1(ra111)* contains a small deletion that removes a portion of exon 2 and the adjacent intron from ORF C18A11.7 removing at least a portion of one immunoglobulin repeat. *dim-1 (ra114)* contains a large deletion that removes the entire C18A11.7 open reading frame. In these blots, DD1 strongly reacts to a 35 KD protein and mildly reacts to an ~95 KD protein against wild type N2 protein extracts. In the case of both deletion mutants, the 35 KD band is completely absent while the 95 KD band is present at the same intensity (Figure 7). Thus, DD1 is specific to the *dim-1* gene product and that product is 35 KD in size. The 95 KB band is probably due to cross reactivity to an unknown protein since it is present in both wild type and mutant blots, and is not of the predicted size of either of the possible *dim-1* protein products. Since both deletion mutants affect ORF C18A11.7, the *dim-1* gene must be encoded by this open reading frame and not C18A11.8. Interestingly, neither deletion mutant affects the upstream open reading frame, C18A11.8, yet no protein of its predicted size is detected on blots with DD1. Since the fusion protein used to generate DD1 contains portions of both open reading frames, we would expect the protein encoded by C18A11.8 to be detected on Western blots as well. However, this is not the case, and therefore we must conclude that either the protein encoded by C18A11.8 is made in such low amounts that it is not detectable by our assay, or that the DD1 antiserum recognizes epitopes specifically encoded by C18A11.7.

### 3.3. Molecular analysis of *dim-1* mutants

To address which portions of the *dim-1* gene are essential to its function and *unc-112* suppression, sequence alterations for six EMS generated *dim-1* alleles, *ra102, ra203, ra204, ra214, ra215, and ra219*, were determined. The entire sequence of both ORF's, C18A11.7 and C18A11.8, were amplified by PCR and sequenced in all six alleles. No sequence alterations were found in the C18A11.8 open reading frame in any of the six
Figure 7. DD1 is specific to the *dim-1* gene product, which is a 35 KD protein.

Purified fusion protein and extracts from N2 and *dim-1* deletion animals were separated by SDS-PAGE, transferred to nitrocellulose and reacted with DD1 GST-depleted antiserum at a 1:5000 dilution.

A. DD1 recognizes the 55 KD GST-fusion protein. DD1 recognizes a 35 KD protein in N2 protein extracts that is missing in *dim-1* deletion alleles, *ral11* and *ra14*. Both deletions affect the downstream ORF C18A11.7. *ral11* is a 183 bp deletion beginning in exon 2 (see Figure 10) and *ra209* deletes the entire coding region.

B. Worms extracts were reacted with DD1 and an affinity purified polyclonal anti-GST serum to recognize GST in the worm as a positive control.
alleles tested. All alterations found within the *dim-1* affect the ORF, C18A11.7. A summary of sequencing results is outlined in Table 1 and Figure 8 shows the approximate positions of the sequence alterations found. All mutations are GC to AT transitions, which is consistent with the specificity of the mutagen used to generate them, EMS. 

*ra219* is a tryptophan to a stop codon in exon 4. *ra102* and *ra215* are identical mutations that affect the splice donor site between exons 1 and 2. *ra203* affects the splice acceptor site between exons 4 and 5, and *ra204* is a tryptophan to stop codon located within exon five (Figure 8). No sequence alteration was found for *ra214* within either ORF. Approximately 20 bp from the far 5’ and 3’ ends of both ORF’s were not covered by these sequence results, nor was any upstream regulatory region. The *ra214* allele is a null mutation (see below) and thus its sequence alteration must reside somewhere within these uncovered regions.

To address whether these sequence alterations result in functional nulls (i.e. complete loss of DIM-1 protein), each allele was analyzed for the presence of any or altered DIM-1 protein by Western blotting using DDI antiserum. No DIM-1 protein was detected in extracts from five of the six *dim-1* alleles on Western blots (Figure 9). Thus, *ra102, ra215, ra203, ra204*, and *ra214* are functional nulls. An approximately 15 KD protein was detected on blots from worms carrying the *ra219* allele, which likely represents a truncated protein product. The *ra219* mutation produces an early stop codon in exon 4, and thus is predicted to encode a 16 KD truncated protein product.
Table 1. *dim-1* mutant alleles.

<table>
<thead>
<tr>
<th><em>dim-1</em> allele</th>
<th>Sequence alteration</th>
<th>Location within C18A11.7</th>
<th>Product formed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ra111</em></td>
<td>183 bp deletion</td>
<td>Exon 2→adjacent intron</td>
<td>None</td>
</tr>
<tr>
<td><em>ra114</em></td>
<td>&gt;2 kb deletion</td>
<td>Entire ORF</td>
<td>None</td>
</tr>
<tr>
<td><em>ra102</em></td>
<td>Splice donor</td>
<td>Exon 1</td>
<td>None</td>
</tr>
<tr>
<td><em>ra203</em></td>
<td>Splice acceptor</td>
<td>Exon 5</td>
<td>None</td>
</tr>
<tr>
<td><em>ra204</em></td>
<td>trp→ stop</td>
<td>Exon 4</td>
<td>None</td>
</tr>
<tr>
<td><em>ra214</em></td>
<td>?</td>
<td>?</td>
<td>None</td>
</tr>
<tr>
<td><em>ra215</em></td>
<td>Splice donor</td>
<td>Exon 1</td>
<td>None</td>
</tr>
<tr>
<td><em>ra219</em></td>
<td>trp→ stop</td>
<td>Exon 5</td>
<td>15 KD truncated product</td>
</tr>
</tbody>
</table>
Figure 8. Positions of the sequence alterations in *dim-1* mutants.

All *dim-1* mutations found lie within a single open reading frame, C18A11.7. A schematic of this ORF is shown and the approximate positions of the mutations found are labeled above. All are GC to AT transitions and affect splicing sites or result in nonsense mutations. The location of the *ra214* mutation was not found therefore, it is absent from this figure.
Figure 9. EMS induced alleles of *dim-1* result in a loss of DIM-1 protein.

Whole worm extracts from N2 and five *dim-1* EMS induced alleles were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with DD1 GST-depleted antiserum at a 1:5000 dilution. Four *dim-1* alleles, *ra102, ra214, ra204,* and *ra203* result in a complete absence of detectable product recognized by DD1. A 15 KD protein is detected in extracts from *ra219*, which is probably a truncated product resulting from the early stop codon found in exon 4. See Figure 10 for the positions of mutations within the *dim-1* gene.
DD1

dim-1 EMS Alleles

N2 ra102  N2 ra219  N2 ra214  N2 ra204  N2 ra203
3.4. **DIM-1 is expressed in the body wall muscle.**

The in situ localization of the *dim-1* protein was determined by indirect immunofluorescence. Mixed stage worms were fixed and stained with DD1 and labeled with fluorescently tagged donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch). Worms were co-stained with various muscle specific monoclonal antibodies to determine what subcellular structures DD1 localizes to. MH24 is a monoclonal antibody that recognizes the protein vinculin, which is found at the base of dense bodies and at the cell margins of body wall muscle (Barstead and Waterston 1989). MH35 recognizes another dense body component, alpha-actinin, which is found throughout the finger-shaped dense body (Francis and Waterston, 1985). Myosin heavy chain A is found at the center of thick filaments and is recognized by DM5.6, while myosin heavy chain B is found at the ends of thick filaments and is recognized by DM5.8 (Miller et al., 1983). Phalloidin recognizes filamentous actin and thus stains the thin filaments in muscle. DD1 was also used to stain worms carrying an *unc-112::gfp* fusion, which is localized to both the dense bodies and M-lines in body wall muscle.

DD1 stains multiple structures in adult worms such as the gonad, vulva, and body wall muscle. The strong reactivity to the gonad was also seen in *dim-1* mutants and in N2 animals stained with pre-immune sera. However, the body wall muscle staining (and perhaps vulva staining) was specific to the DD1 sera and was absent in *dim-1* mutants (data not shown). Thus, DIM-1 localizes to the body wall muscle in adult animals.

The subcellular distribution of DIM-1 within the body wall muscle cells occurs in repeated longitudinal striations spaced approximately one sarcomere length apart (Figure 10). This striated pattern is reminiscent of the pattern observed with myofilaments and their associated proteins (Waterston, 1988). The bands themselves are rather broad and diffuse such as those of myofilaments rather than narrow and compact like the banding pattern of M-lines.
### Table 2. Antibodies used and their specificities.

<table>
<thead>
<tr>
<th>Name</th>
<th>Polyclonal/ Monoclonal</th>
<th>Protein Recognized</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD1</td>
<td>Rabbit polyclonal</td>
<td>DIM-1</td>
<td>Body wall muscle</td>
</tr>
<tr>
<td></td>
<td>Mouse monoclonal</td>
<td>Myosin Heavy Chain A</td>
<td>Thick filaments, central portion, body wall muscle</td>
</tr>
<tr>
<td>DM5.6</td>
<td>Mouse monoclonal</td>
<td>Myosin Heavy Chain B</td>
<td>Thick filaments, lateral portions, body wall muscle</td>
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<tr>
<td>DM5.8</td>
<td>Mouse monoclonal</td>
<td>Alpha-actinin</td>
<td>Dense bodies</td>
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<td>Mouse monoclonal</td>
<td>Vinculin</td>
<td>Dense bodies and cell margins</td>
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<td>Mouse monoclonal</td>
<td>Intermediate Filament Protein</td>
<td>Hemidesmosmes in hypodermis overlying body wall muscle</td>
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<tr>
<td>MH5</td>
<td>Mouse monoclonal</td>
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Figure 10. **DIM-1 is expressed in the body wall muscle.**

Worms were prepared for immunofluorescence by the freeze-crack method (see Materials and Methods) and stained with DD1 at a 1:100 dilution. DD1 stains adult body wall muscle in a striated pattern reminiscent of the pattern of myofilaments in muscle. Note that the staining is rather diffuse and in broad bands rather than in the narrow striations seen in M-line staining patterns (refer to Figure 6 and Figure 14). The striations are one sarcomere length apart, identical to the spacing between adjacent M-lines or dense bodies. Three representative muscle cells are shown.
DIM-1
To determine what filamentous structures DD1 localizes to, animals were stained with monoclonal antibodies against each of the two body wall muscle myosins. DM 5.6 recognizes myosin heavy chain A (MHC A), which makes up the central region of myosin filaments. DM5.8 recognizes MHC B that forms the ends of thick filaments (Miller et al. 1983). In both cases, DD1 consistently showed a striated pattern alternating with that of the thick filaments (Figure 11). The most convincing and conclusive DD1 staining pattern was that which was coupled with DM5.8. DM5.8 stains the ends of thick filaments, which results in a staining pattern of broad striations. The striations contain a narrow central region devoid of staining which corresponds to the region where MHC A and the M-line are found (H-zone). The larger gaps between striations correspond to the location of thin filaments (Figure 11). DD1 is localized to the larger gaps between MHC B and not to the narrow gaps corresponding to the H-zone (Figure 11). Therefore DIM-1 is localized to the region containing thin filaments. To determine if DIM-1 colocalizes with thin filaments, worms were stained with DD1 and FITC conjugated phallodin, which binds filamentous actin. Unfortunately, all attempts to stain with this combination of fluorescent labels were unsuccessful. Phalloidin staining was robust, but DD1 staining was absent. Perhaps there are competitive interactions between DD1 and phalloidin with actin making these two labels incompatible.

Worms were also stained with various other muscle specific markers to confirm where DIM-1 is localized. When worms were stained with membrane-associated and dense body specific markers, location of DD1 staining was less obvious and less consistent. For example, MH24, which recognizes vinculin at the base of dense bodies and cell boundaries, shows a pattern of punctate striations that alternate with DIM-1 containing striations (data not shown). On the other hand, staining with another dense body specific marker MH35, which recognizes alpha-actinin, reveals a pattern where rows of dense bodies overlap with DD1 striations and other areas where rows of dense bodies alternate with DD1 striations (Figure 12). Again, when worms carrying an UNC-112::GFP reporter transgene were stained with DD1, there was no consistent overlap between the two markers at either the dense bodies or the M-lines (Figure 13).
Figure 11. DIM-1 does not colocalize with MHC A, but partially overlaps with MHC B.

A. Worms were stained with DD1 and DM5.8, which recognizes MHC B. A single muscle cell is shown. The arrow indicates a region devoid of DM5.8 staining corresponding to the location of MHC A and the M-line. Note that DD1 staining is found between DM5.8 striations, which corresponds to the location of thin filaments within muscle. Some yellow is observed in the merged image indicating that there is some overlap between the two antigens.

B. Again a single muscle cell is shown stained with DD1 and DM5.6, which recognizes MHC A. DD1 striations alternate with DM5.6 and do not overlap.

C. Schematic of a single sarcomere showing the location of antigens recognized by the antibodies indicated above. Red filaments contain MHC A, light blue filaments contain MHC B, and dark blue filaments are actin containing thin filaments.
Figure 12. DIM-1 and alpha-actinin are not strongly associated.

Worms were stained with DD1 (green) and MH35 (red), which recognizes alpha-actinin in the dense bodies. In panel A, DD1 does not appear to colocalize with the dense bodies (arrows). DD1 striations alternate with rows of dense bodies. However, in panels B and C, DD1 striations line up with dense bodies. This inconsistency may be due to the position of the muscle cell with respect to the plane of view. In any case, DD1 is not tightly associated with alpha-actinin in the dense bodies, or one would expect to find them to colocalize regardless of the orientation of the muscle cell.
3.5. **DIM-1 is not required for UNC-112 localization.**

If the genetic interaction observed between the *unc-112* and *dim-1* genes arises from a physical interaction between their gene products, one might predict the localization of their gene products to be dependent on one another. To determine whether DIM-1 is required for proper UNC-112 localization, an *unc-112::gfp* reporter fusion was introduced into *dim-1(ral02)* mutants. This functional *unc-112::gfp* reporter fusion contains the regulatory and coding sequence of *unc-112* fused to *gfp* and can completely rescue an *unc-112* null mutation. It is localized to dense bodies, M-lines, and cell-to-cell boundaries, the attachment structures within the body wall muscle. Diffuse muscle expression begins in embryos and by the first larval stage UNC-112 is localized into discrete structures corresponding to the dense bodies and M-lines. This expression is maintained throughout adulthood. In adult *dim-1* animals, UNC-112 is properly localized to its position at the muscle cell membrane, but is clearly disorganized. Dense body localization appears fractured, and M-lines are often discontinuous (Figure 14). The severity of disorganization increases with the age of the *dim-1* mutant. To address whether this disorganization is due to initial mislocalization in the absence of DIM-1, or due to a secondary affect of the muscle disruption associated with *dim-1* mutants, UNC-112 localization was examined in *dim-1* larva. UNC-112 distribution in *dim-1* L2, and L3 larvae is virtually indistinguishable from wild type. It is not until *dim-1* animals become late larvae or adults that UNC-112 begins to appear disorganized. Thus, the disorganization of UNC-112 observed in *dim-1* mutants is a result of the general disruption of the myofilament lattice rather than a specific requirement for DIM-1 in UNC-112 localization.
Figure 13. DIM-1 does not colocalize with UNC-112.

Worms carrying an UNC-112::GFP reporter fusion were stained with DDI. DDI staining was found in some cases between M-lines and dense bodies (arrow), or colocalizing with M-lines (arrowhead), or colocalizing with dense bodies (data not shown). Again, this discrepancy is probably due to varying positions of the muscle cell with respect to the plane of view. It can be concluded however, that DIM-1 does not strongly colocalize with UNC-112 thus, they are not likely to be directly associated.
Figure 14. UNC-112 localization in dim-1 (ra102).

The unc-112::gfp (raEx16) reporter gene is expressed at dense bodies, M-lines and cell margins of body wall muscle. A few cells of a single adult muscle quadrant are shown at 63x magnification. In adult dim-1 mutants, UNC-112::GFP is disorganized. The M-lines are discontinuous and the dense bodies are fractured. The extent of disorganization is variable between cells (compare the cell at bottom right to the centered cell in the dim-1 panel) and progressively worsens as the animal ages.
unc-112::GFP

Wild Type

dim-1
Chapter 4. DISCUSSION

4.1. The dim-1 gene corresponds to the ORF C18A11.7.

The sequence of cDNA yk399b7 suggests that the two open reading frames C18A11.7 and C18A11.8 encode a single gene with a large intron rather than two separate genes as predicted by Gene Finder. The 5' end of C18A11.7 contains a classic splice acceptor site (UUUUCAG/R) and is not trans-spliced to the SL1 splice leader, consistent with both ORF's actually encoding a single gene. However, SL1 trans-splicing to the 5' end of transcripts is not an absolute requirement in C. elegans, as approximately 70% of C. elegans genes are trans-spliced to SL1 (Spieth et al., 1993). Therefore it is still possible that C18A11.7 alone encodes a single gene.

The data presented in this thesis suggest that C18A11.7 and C18A11.8 are separate genes and the dim-1 gene corresponds to the downstream ORF, C18A11.7. A polyclonal antiserum generated against the DIM-1 protein recognizes a 35 KD protein, the predicted size encoded by one of the open reading frames, not both. Eight dim-1 mutations were found to affect the C18A11.7 ORF, all of which result in either the complete loss of the 35KD protein or in the case of ra219, a truncated product. It is possible that the 35 KD protein is a degradation product but seems unlikely as protein samples were prepared with varied conditions from mild heating to boiling and showed no difference in protein mobility or binding affinity of the antibody. The complete
coding sequence of C18A11.7 plus 3.5 kb of upstream sequence has been amplified by PCR for microinjection into dim-1 mutant animals. If the C18A11.7 ORF can be shown to completely rescue the dim-1 phenotype alone, we may conclude that the dim-1 gene encodes a 35 KD protein comprised of three immunoglobulin repeats corresponding to the open reading frame, C18A11.7.

What then does the cDNA clone yk399b7 represent? These two ORF's could comprise an unusual operon. Approximately 25% of C. elegans genes are found in operons that are transcribed as polycistronic units (Blumenthal et al., 1997). Usually, genes in an operon are separated by only 100-400 base pairs and cDNAs generated from polycistronic transcripts contain some of this spacer sequence, which is cleaved after poly-adenylation and trans-splicing (Blumenthal et al., 1997). C18A11.7 and C18A11.8 are separated by 3.5 kb and the cDNA, yk399b7, contains none of this spacer sequence. Downstream genes within an operon are normally trans-spliced to the splice leader, SL2 but C18A11.7 is not. Therefore C18A11.7 and C18A11.8 are not likely to comprise an operon.

The cDNA database in which yk399b7 was found contains approximately 20 other clones corresponding to C18A11.7, none of which include any sequence from the upstream gene. Perhaps yk399b7 represents a rare transcript that is cleaved either post-transcriptionally or is a strange artifact produced during the library generation. Northern analysis of total worm mRNA may be the best approach to test these possibilities.

4.2. DIM-1 is localized within adult body wall muscle.
The DD1 antiserum generated against the dim-1 gene product reveals a striated staining pattern in body wall muscle similar to that of myofilament associated proteins. This striated pattern did not colocalize with either isoform of body wall muscle myosin suggesting that DIM-1 may associate with thin filaments.

DD1 did not colocalize strongly with attachment structure proteins that make up dense bodies therefore DIM-1 does not appear to be interact directly with these thin filament anchoring structures. DD1 showed an inconsistent pattern of striations that alternate or align with those of vinculin, alpha-actinin, and UNC-112. These initially confusing results suggest that DIM-1 may be localized to a region of the cell more
internal to UNC-112, vinculin, and alpha-actinin, which are membrane-associated components. Depending on the orientation of the muscle cell with respect to the plane of view, more internal components may appear shifted with respect to those at the membrane. This possibility is further supported by the observation that when UNC-112::GFP transgenic worms are stained with DM5.6, the labeled central thick filaments do not consistently align with M-lines. In fact, similar to the observation with DD1 staining, DM5.6 staining was seen aligning with dense bodies, M-lines, or the area between (Devenport, data not shown). This peculiar observation could be specific to freeze-crack prepared worms, as this procedure does not call for the use of a strong fixative.

These immunofluorescence results are also complicated by the fact that achieving muscle staining with DD1 proved to be difficult as only those muscle cells whose lattice seemed to be partially disrupted from the freeze-crack preparation showed consistent DD1 staining. Evidently, the epitope recognized by DD1 is difficult to access. The other sarcomere components may not show their proper localization in the partially disrupted muscle cells that achieve DD1 staining. Thus, it is difficult to interpret precisely what subcellular structure DIM-1 is localized to from these colocalization studies. However, some conclusions can be made. First, DIM-1 is not tightly associated with the dense body components tested here, or with the M-line. If proteins tightly associate with one another, one would expect them to remain associated, and even become mislocalized together in partially disrupted cells. Second, DIM-1 is found in a pattern that is reminiscent of filament associated proteins. Third, DIM-1 is not associated with the central region of thick filaments and partially overlaps with the ends of thick filaments suggesting that it may be associated with the thin filaments. In light of the \textit{dim-1} phenotype, this localization data suggests a role in filament stability for the DIM-1 protein, perhaps specifically in the stability of thin filaments.

Perhaps the most interesting result revealed by these localization experiments is that DIM-1 does not colocalize with UNC-112::GFP. This result indicates that the genetic interaction between \textit{dim-1} and \textit{unc-112} results from a distant effect caused by the absence of \textit{dim-1} rather than a direct interaction between their gene products.
Suppression may therefore result from a long-range effect on UNC-112 \((r367)\) through a change in myofilament dynamics as a result of the loss of DIM-1.

### 4.3. Muscle proteins of the immunoglobulin superfamily.

Proteins of the Ig superfamily contain protein domains with a general fold that can be described as an antiparallel, Greek-key, beta-sandwich (Bork et al., 1994, Smith and Xue, 1997). This protein module was first discovered in antibodies and is found in a diverse group of cell adhesion molecules, cell surface receptors, and muscle proteins (Smith and Xue, 1997). Ig domains found in muscle proteins often occur in multiple copies within this superfamily of proteins, frequently in tandem repeats (Gregorio et al., 1999, Benian et al., 1996, reviewed in Furst and Gauntel, 1995). Some of the largest proteins reported belong to this superfamily and include the giant muscle proteins titin which contains 166 Ig domains, the 750 KD invertebrate muscle protein, twitchin, and \textit{C. elegans} M-line protein UNC-89 (Labeit et al., 1990, Benian et al., 1989, 1996).

DIM-1 is a member of the immunoglobulin superfamily of proteins of the intracellular muscle branch. The founding member of this branch is the \textit{C. elegans} UNC-22/twitchin (Benian, et al., 1989), which localizes to A-bands (Moerman et al.1988), and is thought to function in the regulation of muscle contraction (Moerman et al., 1982). Since then many intracellular muscle proteins have been identified and found to be members of this subfamily. The vertebrate M-line contains M-protein, myomesin (Furst and Gauntel, 1995), the carboxy-terminal portion of titin (Labeit and Kolmerer, 1995), and skelemin (Price and Gomer, 1993), all of which contain multiple Ig domains and fibronectin type III (FnIII) domains. Other muscle members of this family include insect projectin (Ayme-Southgate et al., 1995), telokin (Gallagher and Herring, 1991), C-protein (Einheber and Fischman, 1990), and kettin (Lakey et al., 1993). All of these proteins including the \textit{C. elegans} proteins, UNC-89 and UNC-22/twitchin, are thought to interact with myosin as well as other proteins through their Ig and fibronectin type III (FnIII) domains (Benian et al., 1989, 1993, 1996). This has been directly demonstrated for titin (Labeit et al., 1992), telokin (Shirinsky et al., 1993), C-protein (Okagaki et al., 1993), and myomesin (Obermann et al., 1997). Two exceptions are kettin, which bind thin filaments and alpha-actinin in insect muscles (Lakey et al., 1993), and a newly described human
muscle protein, myotilin, which directly interacts with alpha-actinin (Salmikangas et al., 1999).

DIM-1 Ig domains fall into the I-type (intermediate) category and are most similar to those found in twitchin, titin, UNC-89, and telokin (Figure 15). Based on this sequence similarity one might expect DIM-1 to be associated with myosin containing A-bands or M-line constituents. However, immunolocalization studies presented here suggest that DIM-1 is associated with the thin filaments. If DIM-1 is associated with thick filaments, it is only at regions that overlap with thin filaments. Interestingly, kettin and myotilin, two muscle Ig superfamily members that interact with actin rather than myosin, contain repeating motifs that fall into the Ig class only (Lakey et al., 1993, Salmikangas, 1999). This is in contrast to M-line and myosin associated Ig superfamily members, which contain FnIII domains as well (Furst and Gauntel, 1995). Like kettin and myotilin, DIM-1 contains only Ig-type repeats. Perhaps this arrangement is an adaptation to binding actin and alpha-actinin rather than myosin (Lakey et al., 1993).

4.4. Suppression of unc-112 (r367) through loss of function dim-1 mutations: a model.

Suppression of the phenotypic effects caused by one mutation through a second mutation in a separate gene suggests the two genes may be involved in the same or a related pathway. Suppression may result from complementary changes in two interacting gene products or indirectly through changes in an alternate pathway (Prelich, 1999). The genetic interaction observed between the unc-112 and dim-1 genes suggests their gene products may be functionally related. Data from this and previous studies indicate that the dim-1/unc-112 interaction is likely to be an indirect one rather than a direct physical association between their gene products.

The dim-1 mutation is a dominant suppressor of unc-112 (r367). Mutations in the dim-1 gene suppress unc-112 well when heterozygous (dim-1+/−) and better when homozygous (dim-1/dim-1) (Gilbert, 1997). A heterozygous deletion covering the dim-1 region also suppresses unc-112 (r367). These earlier results suggested that either reduction or loss of the dim-1 gene product leads to suppression of the unc-112 (r367)
Figure 15. Alignment of DIM-1 to twitchin and titin Ig domains.

Three immunoglobulin repeats of DIM-1 were aligned with three tandem Ig domains in a fragment of *C. elegans* twitchin protein and a fragment of human titin. These two fragments carried the highest homology scores in a BLAST search with DIM-1. Both fragments are 28% identical and 44% similar to DIM-1. Higher identity scores are found when individual Ig domains are BLASTed against each other. The sequence from DIM-1 and twitchin and titin fragments were imported into MacVector, aligned with Clustal, and formatted in SeqVu. The *C. elegans* twitchin sequence is from a 380 amino acid fragment, accession number s57218. The amino acids from the titin fragment are numbered on either side of the alignment.
### Alignment of DIM-1 to twitchin and titin Ig domains

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60
phenotype. The sequence data and Western blot results presented here have confirmed this suggestion. Eight dim-1 alleles have been characterized, including two deletions and six EMS alleles. Western blot analysis revealed that seven dim-1 mutations result in a complete loss of the protein recognized by the DD1 antiserum and thus are null alleles. One, ra219, produces a truncated DIM-1 product, but its phenotype is no different from other alleles and therefore can also be considered a functional null. Because the loss of dim-1 suppresses unc-112 (r367), suppression clearly cannot be due to restoring a physical interaction between their two gene products.

Introduction of the unc-112::gfp transgene into dim-1 homozygous mutants revealed that while unc-112 is disorganized in adult muscle, it appears to be properly localized in dim-1 larvae. Thus, DIM-1 is not required for localizing unc-112 to its proper location in dense bodies and M-lines. Consistent with this result is the finding that DIM-1 appears to be associated with the myofilaments within the sarcomere rather than the attachment structures. Together, these results indicate that the interaction between unc-112 and dim-1 is an indirect one occurring over some distance within the muscle cell rather than a direct association between the two proteins.

How might the loss of function mutations in dim-1 result in the suppression of unc-112? The UNC-112 protein is required during initial muscle assembly where it plays a role in recruiting and stabilizing attachment proteins to form dense bodies and M-lines (Rogalski et al., submitted 1999). When this protein is absent, a myofilament lattice fails to form and animals arrest as two-fold embryos. Loss of DIM-1 cannot suppress this pat phenotype resulting from null mutations in unc-112. On the other hand, mutations in dim-1 can suppress the severe muscle disruption and paralysis resulting from the unc-112 missense allele r367 (Gilbert, 1997). The r367 mutation changes a threonine (105) residue to an isoleucine and is thought to produce an altered protein product (Rogalski, unpublished results). The r367 protein functions normally during initial assembly of sarcomeres and can maintain the lattice during larval growth and movement. However, it cannot maintain muscle integrity beyond the L3 stage, a time during which the myofilament lattice undergoes a significant amount of growth. The myofilament lattice gradually becomes disrupted to the point of causing paralysis in adult animals. Interestingly, it is during this same period of considerable growth that the requirement for
DIM-1 begins and the disorganized muscle phenotype of dim-1 mutants is first seen. Because the disorganized muscle phenotype is first observed during the L3 and L4 stages of development, DIM-1 may not be expressed prior to these stages. Thus the onset of the unc-112 (r367) phenotype correlates with the probable onset of DIM-1 expression.

The DIM-1 protein is not required for assembly of sarcomeres into a myofilament lattice but does play a role in the stability and maintenance of the lattice during growth. DIM-1 appears to have a filament associated distribution in adult muscle and may stabilize the filaments while new proteins are being added to the sarcomeres during growth and contraction. This may add a certain amount of tension to attachment structures that anchor the filaments to the membrane and ECM. Under wild-type conditions this tension presents no serious strain to the lattice scaffold and the addition of new filaments and other muscle components proceeds normally. However, the lesion associated with the unc-112 (r367) mutation may produce a destabilized product that is not able maintain the necessary interactions with other attachment proteins when DIM-1 is present. Therefore the underlying scaffold upon which the lattice is built becomes disrupted and the muscle begins to fall apart. However, if DIM-1 is absent and thus not presenting added strain to attachment structures, the r367 protein may be able to maintain its interactions with other attachment proteins. The muscle is still somewhat destabilized by the absence of DIM-1, and the UNC-112 (r367) interaction is not as strong as wild type hence, the muscle shows an intermediate phenotype between that caused by the dim-1 or unc-112 (r367) mutations alone (see Figure 5). Based on this model, one might expect dim-1 mutations to interact genetically with additional mutations that affect muscle components other than UNC-112. It will be interesting to see if dim-1 mutations can suppress missense mutations in other muscle assembly genes.

4.5. The dim-1 gene is required for myofilament stability.

The elements that are required for initial assembly of the myofilament lattice are not sufficient to maintain its integrity beyond the larval stages. Other elements are necessary. This has been shown for the proteins encoded by the unc-22 (Benian et al., 1989), unc-89 (Benian et al., 1996), and unc-87 (Goetinick and Waterston, 1994) genes, as well as for certain alleles of the unc-52 (Mullen et al., 1999), unc-97 (Hobert et al., 1999), and unc-
genes. Loss of the \textit{dim-1} gene product results in a destabilization of the myofilament lattice. Minor disorganization is first seen during late larval stages and progressively worsens as the animal ages and grows. The extent of disorganization is minor compared to that caused by certain muscle destabilizing mutations such as \textit{unc-54}, \textit{unc-112 (r367)}, or a subset of \textit{unc-52} mutations (Epstein et al., 1974, Rogalski et al., 1995). Worms that carry \textit{dim-1} mutations can move at a rate comparable to wild type thus the lattice must remain considerably organized to maintain contractions that are transduced to movement. Therefore the role \textit{dim-1} plays in maintaining the overall integrity of the myofilament lattice appears to be minor. The stabilizing role of DIM-1 also appears to influence the pattern of sarcomere addition during growth. As mentioned in the introduction, mutations in \textit{dim-1} also cause many sarcomeres to display a ‘chevron’ phenotype. A-bands and I-bands are observed to intersect each other at an acute angle rather than lying in parallel (see figure 5). It appears that DIM-1 may not only stabilize filaments but also affect the angle in which the sarcomeres are oriented as new sarcomeres are added. The localization of DIM-1 suggests that the stabilizing function of \textit{dim-1} acts on the filaments themselves. DIM-1 may hold existing filaments in register as new sarcomeres are built and existing sarcomeres grow. When \textit{dim-1} is absent, the filaments become destabilized and perhaps shifted so that new filaments are sometimes improperly placed. This could lead to a general instability of the myofilament lattice causing an overall disorganization muscle.

Muscle mutations have been previously shown to affect thick and thin filaments to different extents suggesting their gene products associate with one set of filaments or the other. Thin filament affecting mutations such as \textit{unc-60} and \textit{unc-78} cause aggregates of thin filaments to accumulate but the organization of thick filaments is not affected. In contrast worms with mutations in the \textit{unc-15}, \textit{unc-45}, and \textit{unc-89} genes have defects primarily in thick filament organization (reviewed in Waterston, 1988). One might expect mutations in \textit{dim-1} to affect thin filament organization to a greater extent than thick filament organization based on the localization of the \textit{dim-1} gene product. So far, the \textit{dim-1} phenotype has not been shown to affect one type of myofilament more severely than the other. Instead, its stabilizing role seems to affect the overall lattice structure. It
will be interesting to observe whether dim-1 mutations have any enhancing effect on the phenotypes caused by other mutations that specifically affect thin filaments.

4.6. Summary
The myofilament lattice exists as a fine balance of forces, tensions, and strains produced by each of its components. Only when all of the components are present, in the proper amounts, and in their functional conformations can the muscle lattice be maintained as a stable structure. Each force contributed by one component is balanced by the forces contributed by other components thus, when one is disrupted or absent, the effect is transmitted over the entire structure. However, not all components contribute equally to the forces stabilizing the lattice. As with any physical structure, removing or destabilizing components that make up the foundation will have drastic effects on the stability of that structure. On the other hand, removing stabilizing components that are added to the main framework will have a less severe effect on the overall integrity of the structure. Mutations in unc-112, unc-52, and myosin affect the foundation of the myofilament lattice and result in a severe and overall disruption of muscle (Williams et al., 1994, Rogalski et al., 1995, Waterston, 1989). Mutations in dim-1 affect a stabilizing component added the main framework of muscle and result in only a minor disruption in muscle integrity and function. DIM-1 may only play a minor role in stabilizing the myofilament lattice, but its influence on the entire muscle structure can surely be appreciated in light of its effect on unc-112 (r367).
References


### Appendix A. Primers used in this thesis

**dim-1**

![Diagram of dim-1 with primers](image)

<table>
<thead>
<tr>
<th>Number</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence</th>
<th>Number</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yk399-0</td>
<td>forward</td>
<td>5’ATGGCATCGTTAGCACCATT3’</td>
<td>11</td>
<td>yk184-5</td>
<td>forward</td>
<td>5’AAGGAGTGCAGAATCTGCG3’</td>
</tr>
<tr>
<td>2</td>
<td>yk399-1</td>
<td>forward</td>
<td>5’GGCTGATGAAAGTTTCGACG3’</td>
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<td>yk184-12</td>
<td>reverse</td>
<td>5’CTTGATTGTGAGAGCTG3’</td>
</tr>
<tr>
<td>3</td>
<td>yk399-5</td>
<td>forward</td>
<td>5’TGACGGAAAAACGGAGAT3’</td>
<td>13</td>
<td>yk184-6</td>
<td>reverse</td>
<td>5’CGTAAAGCTTCAGGTTGAAG3’</td>
</tr>
<tr>
<td>4</td>
<td>yk399-2</td>
<td>reverse</td>
<td>5’CTGGTTACAGAAGTGGCTT3’</td>
<td>14</td>
<td>yk184-3</td>
<td>forward</td>
<td>5’AAATCATCCAGAGACTG3’</td>
</tr>
<tr>
<td>5</td>
<td>yk399-7</td>
<td>forward</td>
<td>5’CTGGTTACAGAAGTGGCTT3’</td>
<td>15</td>
<td>yk184-2</td>
<td>reverse</td>
<td>5’TCCACCAACAACCTTGGCC3’</td>
</tr>
<tr>
<td>6</td>
<td>yk399-4</td>
<td>reverse</td>
<td>5’CATCAAGTGCTTACCTCTT3’</td>
<td>16</td>
<td>yk184-11</td>
<td>forward</td>
<td>5’GACATTGGAATACCTCCAGC3’</td>
</tr>
<tr>
<td>7</td>
<td>ykint-2</td>
<td>reverse</td>
<td>5’CTCGGTGATAGAGCAGAGC3’</td>
<td>17</td>
<td>yk184-7</td>
<td>reverse</td>
<td>5’CATTTCCCTCGTGTGTTGTC3’</td>
</tr>
<tr>
<td>8</td>
<td>ykint-1</td>
<td>forward</td>
<td>5’GGACTGCTGAGAGCCGATAAT3’</td>
<td>18</td>
<td>yk184-4</td>
<td>reverse</td>
<td>5’AGCCGATGATCCATTGAGTC3’</td>
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<tr>
<td>9</td>
<td>yk184-1</td>
<td>forward</td>
<td>5’ACACTTCCCACAACACGAG3’</td>
<td>19</td>
<td>yk184-10*</td>
<td>reverse</td>
<td>5’GGTCTAGTTACAGGTTCCC3’</td>
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<tr>
<td>10</td>
<td>yk184-8</td>
<td>reverse</td>
<td>5’ATGAGCCATCGGCTTGG3’</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

* yk184-10 is located ~450 bp downstream of the stop codon in the 3’ UTR
## Appendix B. Muscle-affecting genes in *C. elegans*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dim-1</em></td>
<td>minor muscle disorganization</td>
<td>Ig superfamily, small</td>
</tr>
<tr>
<td><em>Deb-1</em></td>
<td>severe Pat</td>
<td>vinculin</td>
</tr>
<tr>
<td><em>Let-802</em></td>
<td>Pat</td>
<td>myotactin</td>
</tr>
<tr>
<td><em>Lev-11</em></td>
<td>mild Twitcher</td>
<td>tropomyosin</td>
</tr>
<tr>
<td><em>Mua-1</em></td>
<td>progressive paralysis</td>
<td>TF, Sp1 family</td>
</tr>
<tr>
<td><em>Mua-2</em></td>
<td>progressive muscle detachment</td>
<td></td>
</tr>
<tr>
<td><em>Mua-3</em></td>
<td>progressive muscle detachment</td>
<td></td>
</tr>
<tr>
<td><em>Mua-4</em></td>
<td>progressive muscle detachment</td>
<td></td>
</tr>
<tr>
<td><em>Mua-5</em></td>
<td>progressive muscle detachment</td>
<td></td>
</tr>
<tr>
<td><em>Mua-6</em></td>
<td>progressive paralysis</td>
<td></td>
</tr>
<tr>
<td><em>Mua-7</em></td>
<td>defective muscle attachment</td>
<td></td>
</tr>
<tr>
<td><em>Mup-1</em></td>
<td>embryonic arrest and detached muscle</td>
<td></td>
</tr>
<tr>
<td><em>Mup-2</em></td>
<td>larval lethal and muscle deformation</td>
<td>troponin T</td>
</tr>
<tr>
<td><em>Pat-2</em></td>
<td>severe Pat</td>
<td>α-integrin</td>
</tr>
<tr>
<td><em>Pat-3</em></td>
<td>severe Pat</td>
<td>β-integrin</td>
</tr>
<tr>
<td><em>Pat-4</em></td>
<td>severe Pat</td>
<td></td>
</tr>
<tr>
<td><em>Pat-6</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pat-8</em></td>
<td>severe Pat</td>
<td></td>
</tr>
<tr>
<td><em>Pat-9</em></td>
<td>severe Pat</td>
<td></td>
</tr>
<tr>
<td><em>Pat-10</em></td>
<td>severe Pat</td>
<td>troponin C</td>
</tr>
<tr>
<td><em>Pat-11</em></td>
<td>mild Pat</td>
<td></td>
</tr>
<tr>
<td><em>Pat-12</em></td>
<td>mild Pat</td>
<td></td>
</tr>
<tr>
<td><em>Act-123</em></td>
<td>(gf) slow to paralyzed, some lethal</td>
<td>actin</td>
</tr>
<tr>
<td><em>Myo-1</em></td>
<td></td>
<td>pharyngeal myosin</td>
</tr>
<tr>
<td><em>Myo-2</em></td>
<td></td>
<td>pharyngeal myosin</td>
</tr>
<tr>
<td><em>Myo-3</em></td>
<td>severe Pat</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td><em>Ace-1</em></td>
<td>Unc as double ace, Let as triple ace</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td><em>Ace-2</em></td>
<td>Unc as double ace, Let as triple ace</td>
<td></td>
</tr>
<tr>
<td><em>Ace-3</em></td>
<td>Unc as double ace, Let as triple ace</td>
<td></td>
</tr>
<tr>
<td><em>Unc-15</em></td>
<td>limp, paralyzed</td>
<td>paramyosin</td>
</tr>
<tr>
<td><em>Unc-22</em></td>
<td>Twitcher</td>
<td>twitchin</td>
</tr>
<tr>
<td><em>Unc-23</em></td>
<td>&quot;benthead&quot;, muscle detachment</td>
<td></td>
</tr>
</tbody>
</table>
Unc-27  sluggish  troponin
Unc-35  loopy, irregular movement  talin
Unc-45  limp, paralyzed, also Pat alleles  unknown ORF
Unc-52  limp, paralyzed, also Pat alleles  perlecan
Unc-54  limp, paralyzed  myosin heavy chain
talin
Unc-60  limp, slow  cofilin family
Unc-68  weak kinker  ryanodine receptor
Unc-78  slow
Unc-82  slow
Unc-87  limp, sluggish  calponin family
Unc-89  moves well  twitchin family
Unc-90  small, rigid paralysis
Unc-93  "rubberband", wild-type null  unknown ORF
Unc-94  slow
Unc-95  slow to paralyzed
Unc-96  slightly slow
Unc-97  limp, paralyzed  5-LIM protein
Unc-98  slow
Unc-105  small, hypercontracted  degenerin family
Unc-109  paralyzed, recessive lethal
Unc-111  moves well
Unc-112  limp, paralyzed, also Pat alleles  MIG-2 homolog
Unc-113  slightly slow
Unc-114  paralyzed
Unc-120  sluggish, paralyzed
Appendix C. *dim-1::GFP* constructs

A. 3.5 kb upstream

B. 3.5 kb upstream

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Appendix C (continued). Construction of \textit{dim-1}:\textit{gfp} reporter constructs

To generate a functional \textit{dim-1}::\textit{gfp} reporter fusion, two approaches were taken. One approach placed the \textit{gfp} coding sequence in an exon near the 3' end of the gene (C18A11.7) and the other placed \textit{gfp} in an exon at the 5' end of the gene (C18A11.7). The entire coding region of C18A11.7 plus 3.5 kb of upstream sequence was amplified using Long Template PCR (Bohringer-Manneheim) with primers yk399-7 (5' GTGCAGAGTTGGAACATCGA 3') and yk184-10 (5' GGTCTAGTAACAGGTGTTCC 3'). The resulting 5.6 kb PCR product was cloned into the pCR2.1TOPO (Invitrogen) vector and subsequently subcloned into pBR322 using BamHI and SphI sites. To construct the first reporter gene, DM#713, pPD18.85 (from Andy Fire 1997 expression vector kit) was digested with SacI to obtain a \textit{gfp} fragment in the proper frame for subsequent subcloning. The 1 kb fragment was gel purified, and subcloned into a SacI site near the 5' end of the \textit{dim-1} gene. The second reporter gene, DM#712, was similarly constructed using a KpnI site near the 3' end of the \textit{dim-1} gene. The \textit{gfp} fragment was obtained from pPD118.90 (from Andy Fire 1997 expression vector kit) which contains \textit{gfp} in the proper frame for digestion with KpnI.