A COMPREHENSIVE RNAi SCREEN FOR NOVEL MUSCLE-AFFECTING GENES IN *CAENORHABDITIS ELEGANS* IDENTIFIES TWO PARALOGS, T04A8.4 AND F36F2.1, HOMOLOGOUS TO MAMMALIAN STARS

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Hon. BSc., The University of Toronto, 2003

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(GENETICS)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2005

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ABSTRACT

One of the fundamental features of metazoan development is myogenesis. A crucial step during myogenesis is the assembly and anchorage of the sarcomere, the essential repeat unit responsible for muscle contraction. In *Caenorhabditis elegans*, four phenotypic classes of muscle mutants defective in some aspect of muscle structure and function have been identified in mutagenesis screens: the uncoordinated (*unc*) class, typified by uncoordinated, slow or no movement, the muscle positioning defective (*mup*) class, the late embryonic lethal class known as paralyzed and arrested at two-fold stage (*pat*) mutants, and the class where animals are capable of wildtype development and movement but have disorganized muscle (*dim*).

Using SAGE and microarray chip analysis we have identified 3395 non-ribosomal/non-mitochondrial genes expressed in muscle. Using an RNAi feeding library, we screened this ‘muscle expressome’ for genes affecting sarcomere assembly, stability and/or function. Worms harboring an extrachromosomal array containing a myosin heavy chain gene, *myo-3*, fused in frame to green fluorescent protein (GFP) were fed bacteria expressing dsRNA corresponding to each gene within the muscle expressome. The progeny of these worms were examined for both overt phenotypes and mislocalization of *myo-3::GFP*. This approach proved to be a rapid and sensitive means to identify genes required to organize sarcomeric proteins into a highly ordered myofilament lattice and we identified 296 genes with defects in *myo-3::GFP* localization in an initial screen and reconfirmed 121 of those genes in a rescreen. RNAi treated animals display an array of myofilament disruptions ranging from small aggregations of *myo-3::GFP* to large
deposits, often accompanied by disorganization of the myofilaments. The high percentage of tested genes affecting muscle sarcomeres, 3.6%, likely reflects the fact that we have already enriched for genes expressed in this tissue. Many of the genes we have uncovered in this screen have human homologs for which little or nothing is known. Two paralogous proteins, T04A8.4 and F36F2.1, identified here share considerable homology to the human protein striated muscle activator of Rho signaling (STARS). I demonstrated that these proteins are expressed in body wall muscle and are required for proper actin and myosin localization in muscle. In addition, T04A8.4 appears to form a synthetic genetic interaction with *mua-6* and *unc-22* and F36F2.1 may form a genetic interaction with *cdc-42*. 
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<th>Description</th>
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<tr>
<td>AMP</td>
<td>ampicillin</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix protein</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Carb</td>
<td>Carbenicillin</td>
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<tr>
<td>CeSTARS</td>
<td>C. elegans homologs of mammalian STARS</td>
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<tr>
<td>dim</td>
<td>disorganized muscle</td>
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<tr>
<td>dsRNA</td>
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<td>ECM</td>
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<td>FACS</td>
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<td>FERM</td>
<td>band F ezrin-radixin-moesin homology domains</td>
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<td>fluorescence recovery after photobleaching</td>
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<td>green fluorescent protein</td>
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<td>high penetrance phenotypic class</td>
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<td>intermediate penetrance phenotypic class</td>
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<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
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<td>Kb</td>
<td>kilobase pair</td>
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<td>KOG</td>
<td>cluster of orthologous groups</td>
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<td>mSTARS</td>
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<td>NGM</td>
<td>nematode growth medium</td>
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<tr>
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<td>open reading frame</td>
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<td>pat</td>
<td>paralyzed and arrested at twofold stage</td>
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<td>serum response factor</td>
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<td>striated muscle activator of Rho signaling</td>
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ACKNOWLEDGMENTS

I would like to take this opportunity to thank my collaborators Barb Meissner and Adam Warner for their help with this project. This project wouldn’t have been possible without the whole ‘screen team’. In addition, I would like to thank our database manager, Adam Lorch, for his excellent work in creating and maintaining the RNAi database.

A special thanks to Dr. Don Moerman for taking on a molecular biology neophyte such as myself, and for allowing me the freedom to decide the direction of my project. I appreciate the friendly, supportive work environment he has created filled with intelligent, amicable coworkers.

I would also like to thank the other members of my committee, Drs. Linda Matsuuchi and Don Riddle for their help and recommendations on this project. This work was supported by grants to Dr. Don Moerman from the National Sciences and Engineering Council (NSERC) and the Canadian Institute for Health Research (CIHR).
CO-AUTHORSHIP STATEMENT

The research reported in this thesis was developed and undertaken in collaboration with Dr. Barbara Meissner and Adam Warner. Both Dr. Meissner and myself initially developed the RNAi screen for novel muscle-affecting genes, but throughout the evolution of the project all three members of the project contributed equally in both labor and ideas.

The second portion of my thesis concerning the two STARS-like proteins, T04A8.4 and F36F2.1, was my own undertaking. All ideas and data analysis were implemented on my own.
CHAPTER I

INTRODUCTION

The assembly of myofibrils into a highly ordered lattice anchored to the sarcolemma is an intricate event involving the coordinated activity of many proteins. In humans, mutations in several sarcomere and sarcolemmal proteins have been shown to cause muscular dystrophy and cardiomyopathies (Itoh-Satoh et al., 2002). For instance, the loss of Kindlin 1, an actin-ECM linker protein first described in *C. elegans* as UNC-112 (Rogalski et al., 2000), causes Kindler syndrome, characterized by neonatal blistering, sun sensitivity, muscle atrophy, abnormal pigmentation, and fragility of the skin (Siegel et al., 2003). Similarly, small deletions in the giant sarcomeric protein titin can lead to a progressive disorder called tibial muscular dystrophy (TMD) in heterozygous patients, or a more severe limb-girdle muscular dystrophy in homozygous patients (Udd et al., 1992). This devastating disorder is characterized by progressive proximal muscular dystrophy, often accompanied by pseudohypertrophy of the calves, which ultimately culminates in death. Clearly, an understanding of muscle development and function is an important step towards the development of treatments for muscle-related disease.

*C. elegans* is an ideal model system in which to study *in vivo* sarcomere assembly and adhesion complex formation since muscle proteins tend to be highly conserved. Information obtained from the study of muscle in nematodes can be extrapolated to other biological systems, including mammals (Epstein et al., 1974; reviewed in Waterston, 1988; Moerman and Fire, 1997). Mutant animals with severe defects are often viable and
fertile. Even if paralyzed, growth on *E. coli* covered agar plates requires little or no movement (Waterston, 1988). Since *C. elegans* are capable of self-fertilization, mutations that inhibit mating do not prevent propagation. In addition, the availability of a sequenced genome (*C. elegans* Sequencing Consortium, 1998) and extensive amounts of expression data (Hill et al., 2000; McKay et al., 2003; David Miller, unpublished) provides researchers with valuable tools to characterize myofibrillogenesis, the assembly of the sarcomere proper. Information collected from *C. elegans* can then be applied to other systems, such as humans, which may aid in treating disease.

1.1. Muscle structure and function

In *C. elegans* there are two major muscle types: the striated body wall musculature and the single-sarcomere, non-striated pharyngeal muscle cells (Epstein et al. 1974). Several other non-striated muscle cells are present in nematodes, including the intestinal contraction muscles and the muscles that control defecation, the anal depressor and sphincter muscles (Waterston, 1988). The most important set of muscles in *C. elegans* are the body-wall muscles. These 95 muscle cells are arranged in four lateral quadrants, with each band containing 24 mononucleate cells, with the exception of the ventral left quadrant that lacks one cell relative to its neighbors (Sulston & Horvitz, 1977). Each body-wall muscle cell resembles an elongated spindle that lies flattened basally against the hypodermis and laterally against a neighboring muscle cell (Hresko et al. 1994). Lying just below the sarcolemma, the muscle cell membrane, is the contractile apparatus responsible for generating the force required for movement and feeding (Fig.1).
Fig. 1. Model of *C. elegans* muscle. An adult worm with body wall muscle cells illustrated (left). A cross-section through the worm with the cuticle, hypodermis and basal lamina peeled away to show the basal surface of 2 muscle cells (center). A high resolution longitudinal section through a muscle cell revealing the structure of the contractile apparatus (right).

*Courtesy Don Moerman*
The myofilament lattice of *C. elegans* is functionally and structurally analogous to the mammalian sarcomere, the essential repeating contractile unit of a muscle cell. In addition, the structures responsible for anchoring the sarcomere to the cell membrane are analogs of mammalian adhesion complexes (reviewed in Moerman and Williams, 2005). The sarcomere consists of thick myosin filaments interdigitated with actin-containing thin filaments, all held in register by a series of accessory proteins. The thick and thin filaments are anchored to the cell membrane via dense bodies (Z line analogs in mammalian muscle) and M lines respectively (Francis and Waterston, 1985). Force is transmitted laterally from muscle via the hypodermis to the overlying cuticle. This transduction of force is mediated by integrin-containing transmembrane receptors. These lie basally to hemidesmosome-like structures in the hypodermis (Bartnik et al. 1986, Francis and Waterston, 1991). The integrins, in turn, associate with intermediate filament-like proteins (Fig. 2).

There are some features of nematode sarcomeres that differ from their mammalian analogs. First, mammalian muscle is cross-striated, whereas nematode muscle is obliquely striated. This results from the fact that adjacent myofilaments are offset from one another by over one micron, or 6° (Mackenzie and Epstein, 1980). This contrasts to mammalian muscle where neighboring units are aligned parallel to one another. Another feature that differs between nematodes and mammals is the composition and size of thick filaments. Although both mammalian and nematode thick filaments contain myosin, nematodes also contain an additional protein, paramyosin (Epstein et al. 1993). This protein acts as the core of the myofilament around which myosin assembles.
Fig. 2. Structure of the *C. elegans* muscle sarcomere. A schematic diagram revealing the location of several different muscle attachment proteins. α-actinin and vinculin are found exclusively at dense bodies, whereas UNC-89 is found only at the M line.

*Courtesy Don Moerman*
Paramyosin/UNC-15 is highly similar to myosin heavy chain rods, except that they do not contain heads, which allows for tight packing of the paramyosin rod with myosin (Kagawa et al., 1989). Nematode thick filaments, at 10uM, are significantly longer than their 1.6 uM vertebrate counterparts (Mackenzie and Epstein, 1980). A final difference between mammalian and nematode muscle pertains to the mode of attachment of the myofilament lattice to the cell membrane and the conductance of tension across the membrane outwards to the cuticle. The ends of nematode muscle cells contain attachment plaques analogous to vertebrate plaques found at myotendinous junctions of skeletal muscle and intercalated discs in cardiac muscle. However, these structures are not the primary means of transmitting tension between cells. The majority of the force is transferred to the cuticle via lateral attachments between the sarcomere and the basal membrane (Francis and Waterston, 1985). Despite the small differences that exist between mammalian and nematode muscle, C. elegans continues to be an excellent model system to study muscle structure and function.

1.2. Myogenesis

Vertebrate skeletal myogenesis is largely dependent upon the MyoD family of basic helix-loop-helix (bHLH) transcription factors. In the absence of MyoD and Myf-5, two of the myogenic regulatory factors (MRFs), no skeletal muscle forms because of a complete absence of myoblast cells (Rudnicki et al. 1993). It is these two MRFs that activate two closely related bHLH transcription factors, MRF-4 and Myogenin (McKinsey et al., 2001). The outcome of this transcriptional cascade is the expression of muscle specific gene products, such as sarcomeric myosin and actin.
In *C. elegans*, the striated body wall muscles are analogous to the skeletal muscle of vertebrates. As is the case in vertebrates, invertebrate bHLH transcription factors of the MyoD family are at least partially responsible for initiating the conversion of myoblasts into terminally differentiated muscle cells (Harfe et al., 1998; Fukushige & Krause, 2005). However, *C. elegans* has a single characterized bHLH protein, CeMyoD (HLH-1). *hlh-1* is zygotically expressed in muscle precursor cells beginning at the 28 cell stage of embryogenesis in specific blastomeres destined to become body wall muscle (Krause et al., 1990). Animals homozygous null for CeMyoD complete embryogenesis, but exhibit extensive postembryonic defects, including body morphology defects, uncoordinated movement and often larval lethality (Chen et al., 1992; Chen et al., 1994). Interestingly, *hlh-1* null mutants have all 81 embryonically-derived muscle cells, all of which express muscle marker genes (Fukushige and Krause, 2005). This implies that there are likely other factors that contribute to making muscle in *C. elegans*.

### 1.3. *C. elegans* muscle attachments

*C. elegans* muscle is arranged such that tension generated from contraction of the sarcomere is transmitted longitudinally to attachment plaques and laterally to muscle-hypodermal structures. The latter structures, homologous to mammalian focal adhesions (FA), convey the majority of the tension between cells. These FA-like attachment structures are formed when the extracellular matrix (ECM) receptor, composed of a heterodimer of αPAT-2/βPAT-3 integrin, becomes concentrated at the basal cell membrane adjacent to the hypodermis where it interacts, either directly or indirectly, with UNC-52/perlecan (Gettner et al., 1995; Francis and Waterston, 1991; Rogalski et al.,
The unc-52 gene encodes a heparan sulfate proteoglycan, homologous to the human protein Perlecan (Rogalski et al., 1993; Noonan et al., 1991) that acts during embryogenesis in concert with the integrin-containing dense bodies and M lines to direct the assembly of the myofibrillar apparatus (Rogalski et al., 1993). In the absence of UNC-52/perlecan all subsequent steps of myofibrillogenesis are blocked (see Fig. 2).

βPAT-3/integrin colocalizes to the membrane with UNC-112/Mig-2, a novel intracellular protein of the FERM family of proteins, a group of proteins with a membrane-binding domain involved in localizing proteins to the plasma membrane (Rogalski et al., 2000). UNC-112 is indispensable for β-integrin localization, but is not required for UNC-52/perlecan organization (Rogalski et al., 2000). Appropriate recruitment of UNC-112 to attachment structures is mutually dependent upon PAT-4/integrin-linked kinase (ILK), a protein that functions both as a kinase and as an adaptor (MacKinnon et al., 2002). Both proteins are also required for the recruitment of PAT-6/actopaxin, a protein required to recruit UNC-89 and myofilaments to nascent attachments (Lin et al., 2003).

DEB-1/vinculin is a cytoskeletal adaptor protein located distally to the membrane that function's in the attachment of actin filaments to the sarcolemma (Barstead and Waterston, 1989, 1991). This protein is recruited to the dense body independent of UNC-112, PAT-4 and PAT-6; however, removal of any of these proteins prevents the normal recruitment of actin filaments to the basal sarcolemma (Barstead and Waterston, 1991; MacKinnon et al., 2002).
Several other adherens junction proteins are present at muscle attachment structures. An important class of proteins, the LIM proteins, so-called because of the presence of LIM domains, play pivotal roles at the integrin-containing attachment sites. LIM domains are histidine/cysteine rich motifs defined by the presence of two zinc-binding regions that act as interfaces for protein-protein interactions (Schmeichel and Beckerle, 1994; Feuerstein et al., 1994; Dawid et al., 1998; Hobert et al., 1999). LIM domain-containing proteins are diverse in location and function. Some of these proteins contain only LIM domains whereas others contain one to a few such motifs as well as other functional domains (Dawid et al., 1998; Hobert et al., 1999).

In *C. elegans* muscle there are at least three critical LIM domain proteins, UNC-95, UNC-97 and UNC-98 (Broday et al., 2004; Hobert et al., 1999; Mercer et al., 2003). Null mutations in any of these genes results in defective myofilament organization and, in the case of UNC-97, embryonic lethality (Norman et al., unpublished results). Genetic studies have implicated these proteins in sarcomere assembly and attachment, but it is important to note that all three of these LIM domain proteins also are found in the nucleus throughout muscle development (Broday et al., 2004; Hobert et al., 1999; Mercer et al., 2003). This may indicate that these proteins are acting as biosensors, possibly controlling muscle gene expression in response to changes in stress and/or the recycling of muscle proteins (Kadrmas and Beckerle, 2004).

### 1.4. An RNAi screen for novel muscle-affecting genes in *C. elegans*

In *C. elegans*, several proteins have been identified that function either within the sarcomere proper or within the adhesion complex responsible for anchoring the
sarcomere to the basal cell membrane. Defects in these proteins often result in strong, visible phenotypes including the pat (paralyzed and arrested at two-fold stage) mutants (Vinculin/DEB-1, UNC-45, MYO-3) (Barstead and Waterston, 1991; Venolia and Waterston, 1990, Miller et al., 1986), the mup (muscle-positioning defective) mutants (Troponin T/MUP-2) (Myers et al., 1996), and the unc (uncoordinated) mutants (PINCH/UNC-97, UNC-95) Hobert et al., 1999; Broday et al., 2004). Alleles of another gene class, the dim (disorganized muscle) mutants (DIM-1), exhibit a far less acute phenotype. These animals are normally phenotypically wildtype, but display minor muscle defects upon close observation (Rogalski et al., 2003).

Detection and analysis of muscle mutants using a conventional chemical mutagenesis approach formed the foundation for much of the research on muscle in C. elegans for the last 20 years. These mutants have been instrumental in elucidating the mechanism behind muscle function and maintenance. As such, the goal of this thesis was to expand our knowledge of muscle structure and function by identifying novel proteins in muscle that function to maintain the integrity of the sarcomere.

It is evident from transcriptional and translational fusion analysis, as well as expression data (McKay et al., 2003), that more proteins function in muscle than have been identified. Conventional mutagenesis screens appear to be approaching saturation. Thus, it became necessary to design a more sensitive screen to detect new muscle mutants. Here we describe a novel, high-throughput RNA interference (RNAi) screen for genes that affect the integrity of myofilaments in C. elegans. RNAi was selected since it is a rapid and reliable means to target the knockdown of a selected gene product and identify the effects of loss-of-function on the whole organism.
This approach has successfully identified 121 genes that are required to maintain the integrity of the myofilament lattice. In addition, we have found visible, overt phenotypes for 21% (717) of all screened genes within the muscle expressome, a comprehensive set of genes shown to be expressed in muscle by serial analysis of gene expression (SAGE) and DNA microarrays. Furthermore, we uncovered 149 essential genes within the muscle expressome, 19 of which elicit apparent pat phenotypes. This represents the first time that RNAi has been performed on the majority of genes within a specific tissue. The RNAi phenotypes we observed should prove to be valuable in the elucidation of gene function when combined with other functional approaches.

Two paralogous proteins, T04A8.4 and F36F2.1, identified in this screen and confirmed to be expressed in body wall muscle by molecular reporter proteins share a high degree of similarity to the mammalian protein STARS (striated muscle activator of Rho signaling). This protein localizes to the I-band in cardiac and skeletal muscle sarcomeres where it may play a significant role in cardiac hypertrophy through its interactions with other proteins during myofibrillogenesis (Arai et al., 2002). The mSTARS protein has been implicated in the promotion of F-actin polymerization through a mechanism that involves the stimulation of the transcription factor serum response factor (SRF) mediated by Rho GTPase. I have shown that the abatement of endogenous mRNA corresponding to either one of T04A8.4 or F36F2.1 gives a similar disorganized myofilament phenotype characterized by discontinuities in myofilaments. Moreover, phalloidin-staining of T04A8.4(gk355) mutants reveals an analogous phenotype with characteristic gaps in the longitudinally arranged bundles of actin filaments. T04A8.4(gk355) worms are significantly longer than wildtype worms and also appear to
be somewhat less motile than N2 worms. I also show that synthetic genetic interactions exist between T04A8.4 and the giant sarcomeric protein unc-22/Twitchin, as well as the hypodermal intermediate filament protein mua-6. It also seems likely that F36F2.1 forms a synthetic interaction with the Rho GTPase cdc-42. The data obtained and discussed in this thesis suggest that the function of the STARS protein may be conserved in C. elegans. Thus, study of these proteins may help elucidate the role of STARS in myofibrillogenesis and cardiac hypertrophy in mammals.
CHAPTER II

MATERIALS & METHODS

2 RNAi Screen for Novel Muscle-Affecting Genes

2.1 RNAi feeding library

The *C. elegans* feeding library used in this screen was purchased from the mammalian genome collection (MRC) Geneservice (U.K.) and was provided by Julie Ahringer’s group at the Wellcome Trust/Cancer research institute at the University of Cambridge, Cambridge (U.K.). The feeding library was constructed by PCR amplifying genomic fragments using Research Genetics Genepairs®. These amplicons were then cloned into the EcoRV site of the L4440 (pPD129.36) vector from Timmons and Fire (1998) and transformed into *E. coli* strain HT115(DE3) (Timmons et al, 2001). Clones were then transferred to 384-well plates. The entire feeding library contains ~17,000 clones and covers 87% of the *C. elegans* genome.

2.1.2 Muscle ‘Expressome’ Construction

A list of genes determined to be expressed in fluorescence-activated cell sorting (FACS)-sorted muscle cells was compiled by Kim Wong at the British Columbia Genome Sciences Center (BCGSC) using expression data from SAGE (data can be viewed at http://tock.bcgsc.bc.ca/cgi-bin/sage140) (McKay et al, 2003) and DNA microarrays (R. Fox and D. Miller, unpublished). To be qualified as a muscle gene, a gene had to be present in either or both SAGE replicates and two of three microarray
experiments. Using these parameters we have generated a comprehensive list of ~4200 genes that are expressed in muscle. After removing ribosomal and mitochondrial genes from the list, the muscle expressome contains 3395 genes. These genes were then individually picked from the Ahringer RNAi feeding library into 96-well plates. All genes that were positive for muscle defects during the screen were picked into 1.5 mL freezer tubes containing 33% glycerol and stored at -80°C.

2.1.3. Experimental Procedure

2.1.3.1 Strains used

All RNAi experiments for the muscle screen were conducted using *C. elegans* strain RW1596 (a gift from P. Hoppe, Dept. of Biological sciences, Western Michigan University, USA). This strain, *myo-3*(st386) V; stEx30, carries a rescuing extrachromosomal array [Pmyo-3::GFP + rol-6(su1006)] with a wild-type copy of *myo-3* fused in frame to green fluorescent protein (GFP). In the confirmatory round of screening, RW1596 worms carrying an extrachromosomal array and a strain (not yet named) with an integrated array were used. These strains were screened in parallel. The latter was used to more efficiently screen for *Pat* embryos, however an examination of the myofilaments of this integrated strain revealed significant defects in the lattice structure and therefore this strain was no longer used.

2.1.3.2 Preparation of worms and bacteria for RNAi

*C. elegans* strain RW1596 worms from two 150 x 15 mm enriched nematode growth medium (NGM) plates streaked with OP50 were rinsed into 10 mL M9 buffer and
treated with hypochlorite solution (75 % dH2O, 20 % sodium hypochlorite, 5 % 10 N KOH) until no carcasses were visible. Embryos were rinsed three times in M9 buffer to remove residual sodium hypochlorite solution and subsequently transferred in 10 mL M9 buffer to a fresh 15 mL Falcon tube. Embryos were permitted to hatch overnight in M9 buffer to synchronize worms at L1. Concurrently, RNAi plates (NGM + 1 mM IPTG + 50 ug/mL carbenicillin) were seeded with 70 uL overnight bacterial culture from each clone in the muscle expressome. The bacteria were allowed to grow overnight to begin expressing dsRNA. The following morning, synchronized L1 worms (designated P₀) were transferred to the streaked RNAi plates. The P₀ generation was allowed to develop for 60-68 hours at 20°C on the RNAi plates, at which time four worms from each plate were transferred onto two fresh RNAi plates (two worms per plate). The screening is done in duplicate to reduce the likelihood of false-positives in the phenotypic and myofilament screens. The cloned P₀ worms were allowed to deposit eggs on the fresh RNAi plates overnight (~18 hours), at which time the P₀ worms were removed. Embryos were allowed to hatch and develop to L4/young adult stage (36-48 hours) (see Fig. 3).

2.1.3.3 RNAi screening of essential genes

RNA-mediated interference of several essential genes resulted in strong embryonic lethality, sterility, larval lethality or larval arrest phenotypes. Because there were no (or very few) worms to score these genes were screened by modifying the RNAi protocol to ensure that some L4/young adult worms would be available for screening. Briefly, synchronized L1 worms were plated onto RNAi plates seeded with L4440 containing HT115(DE3) bacteria. These worms were incubated at 20°C until they had
Fig. 3. RNAi screen for novel muscle mutants in *C. elegans*. In brief, a list of genes shown to be expressed in muscle by SAGE and microarrays was screened using an RNAi feeding library. Bacterial clones expressing dsRNA corresponding to each gene in the muscle expressome were fed to *C. elegans* strain RW1596. RNAi-treated animals were scored for visible phenotypes and myofilament defects.
matured to adult stage and begun producing eggs. Two adult worms were transferred to the essential gene’s RNAi plates and allowed to lay eggs overnight (~18 hours). The following day P₀ worms were removed from the plates. F1 worms were allowed to develop to L4/young adult at which time they were screened using a Zeiss Axiophot compound fluorescent microscope under 200x magnification. Digital photos were taken using a Qimaging QICAM digital camera running Qcapture version 1.68.4 under 400x magnification.

2.1.3.4 Overt phenotypic screening

Prior to the myofilament screen, worms are screened for a variety of gross anatomical defects, and for embryonic lethality. If a plate had a large number of unhatched embryos (≥25% of total animals on the plate), then the gene was said to cause embryonic lethality. Unhatched embryos were further examined to determine whether they were pat embryos. Post-embryonic RNAi phenotypes were analyzed for sterility, uncoordinated movement, growth defects, body morphology defects, lineage defects (i.e. missing muscle quadrants), blistering along the cuticle, clear patches, dauer formation, dumpy, sluggish or paralysed movement, egg laying defects, abnormally high incidences of males, adult lethality, hyperactive behavior, abnormal social behavior and more (see Table 1). All phenotypic screening was accomplished using a Wild Heerbrugg dissecting microscope.
Table 1. Overt phenotypes observed in the RNAi screen. A list of the gross RNAi phenotypes scored in the muscle expressome screen and the total number of clones displaying that RNAi phenotype.

<table>
<thead>
<tr>
<th>Term</th>
<th>Phenotype</th>
<th># Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal social behavior (abs)</td>
<td>clumping/grouping of worms</td>
<td>2</td>
</tr>
<tr>
<td>Adult lethal (adl)</td>
<td>Adult lethality</td>
<td>9</td>
</tr>
<tr>
<td>Blistered (bli)</td>
<td>Blistering of the cuticle</td>
<td>2</td>
</tr>
<tr>
<td>Body morphology defect (bmd)</td>
<td>Anomalous body defects</td>
<td>6</td>
</tr>
<tr>
<td>Clear (clr)</td>
<td>Clear patches</td>
<td>1</td>
</tr>
<tr>
<td>Dauer formation (daf)</td>
<td>Abnormal dauer formation</td>
<td>3</td>
</tr>
<tr>
<td>Dumpy (dpy)</td>
<td>Swelling of the posterior end</td>
<td>24</td>
</tr>
<tr>
<td>Egg laying defects (egl)</td>
<td>Defective/delayed egg laying</td>
<td>2</td>
</tr>
<tr>
<td>Embryonic lethality (emb)</td>
<td>Arrest during embryonic lethality; not at twofold stage of development</td>
<td>149</td>
</tr>
<tr>
<td>High incidence of males (him)</td>
<td>Abnormally high incidence of males</td>
<td>2</td>
</tr>
<tr>
<td>Hyperactive (hyp)</td>
<td>Abnormally active when stimulated</td>
<td>4</td>
</tr>
<tr>
<td>Larval arrest (lva)</td>
<td>Developmental arrest at L1-L4 stage</td>
<td>66</td>
</tr>
<tr>
<td>Larval lethal (lvl)</td>
<td>Lethality of larval worms</td>
<td>8</td>
</tr>
<tr>
<td>Lineage defective (lin)</td>
<td>Abnormal or missing cells</td>
<td>23</td>
</tr>
<tr>
<td>Long body (lon)</td>
<td>unusually long body</td>
<td>1</td>
</tr>
<tr>
<td>Molt defect (mlt)</td>
<td>Larval molting defective</td>
<td>0</td>
</tr>
<tr>
<td>Multi vulva (muv)</td>
<td>Occurrence of multiple vulvae</td>
<td>0</td>
</tr>
<tr>
<td>Paralyzed (prz)</td>
<td>Very sluggish or no movement</td>
<td>3</td>
</tr>
<tr>
<td>Paralyzed and arrested at twofold stage (pat)</td>
<td>Exploded worms at the twofold stage of embryogenesis</td>
<td>19</td>
</tr>
<tr>
<td>Ruptured (rup)</td>
<td>Unhealthy worms</td>
<td>0</td>
</tr>
<tr>
<td>Sick (sck)</td>
<td>Unhealthy worms</td>
<td>38</td>
</tr>
<tr>
<td>Slow growth (gro)</td>
<td>Slow growth relative to control plates</td>
<td>241</td>
</tr>
<tr>
<td>Sluggish (slg)</td>
<td>Slow movement</td>
<td>32</td>
</tr>
<tr>
<td>Small (sma)</td>
<td>Smaller than control-plate worms</td>
<td>2</td>
</tr>
<tr>
<td>Small brood size (sbs)</td>
<td>Small brood size relative to control plates</td>
<td>47</td>
</tr>
<tr>
<td>Sterile (ste)</td>
<td>Sterile worms; few, if any, progeny</td>
<td>81</td>
</tr>
<tr>
<td>Uncoordinated (unc)</td>
<td>Uncoordinated movement</td>
<td>170</td>
</tr>
<tr>
<td>Vulvaless (vul)</td>
<td>Lack of vulval muscle</td>
<td>0</td>
</tr>
</tbody>
</table>
2.1.3.5 Age-related decline in myofilament integrity assay

RW1596 worms were screened for age-related sarcopenia by growing this strain on L4440 containing HT115(DE3) bacteria. Worms were screened for myofilament defects at L4/young adult, day 1 adult and day 2 adult stages using a Zeiss Axiophot compound fluorescent microscope (D-7082 Oberkochen) under 200x magnification. Digital photos were taken using a Qimaging QICAM digital camera running Qcapture version 1.68.4 at 400x magnification.

2.1.3.6 Myofilament-anatomy screening

A random subset of worms from each duplicate RNAi plate were picked into 15-uL M9 + 10 % sodium azide on a glass microscope slide. A coverslip was gently laid on top the worms and the slide was quickly mounted under a Zeiss Axiophot compound fluorescent microscope (D-7082 Oberkochen) and screened under 200x magnification for disorganized myofilaments. In the preliminary screen, a minimum of 10-20 worms were screened for myofilament irregularities. The number of worms scored for myofilament defects in the rescreen was increased to a minimum of 20 and a maximum of 40. Each worm's myofilaments were then screened for the following disruptions: local GFP aggregations within the myofilament, general GFP masses that fall adjacent to the filaments, discontinuities in the fibres, generalized disorganization of the myofilaments and/or dim GFP expression (Fig. 4). Digital photos were taken using a Qimaging QICAM digital camera running Qcapture version 1.68.4 under 400x magnification. A gene was classified as ‘muscle-affecting’ if ≥50% of worms on a slide exhibited some combination of the aforementioned phenotypes.
Fig. 4. Observed myofilament defects. A range of myofilament irregularities were observed and categorized in the RNAi screen. In (A), general disorganization of the myo-3::GFP reporter is evident. GFP aggregates were often found adjacent to the filaments (B) or along the length of the myofilaments (C). Also observed were animals that had depleted GFP intensity (D). Scale bar = 50 μm
2.1.3.6.1 PCR confirmation of positive RNAi clones

To confirm the presence of the proper RNAi clone, PCR confirmation using gene
specific primers for all genes within the (+/+) and (-/+) classes (see results for
explanation) was performed by picking a single bacterial colony from the screened gene
into 50µL of PCR mix (1x Thermopol buffer, 480 µM deoxynucleotidetriphosphates, 10
pmol forward primer, 10 pmol reverse primer, 1 U Thermopol Taq). PCR products were
separated by gel electrophoresis on a 1% agarose gel in 1X Tris-Acetic acid-EDTA
(TAE) running buffer. Visualization of separated products was accomplished using a UV
transilluminator.

2.1.3.7 Construction of an RNAi database for information storage

A database to keep track of the RNAi observations was set up using MySQL
3.23.49, with a web interface written in PHP 4.3.4, both hosted on a PowerMac G5
running Darwin 7.8.0. This was used to automatically generate statistics on the worm, to
retrieve records of myofilament observations, and to search across data fields such as
gross phenotype, cosmid, well location, and description. This database was developed in
collaboration with Adam Lorch (Computer technician, Moerman lab, University of
British Columbia, Canada).

2.1.3.8 Construction of RNAi clones

Construction of the GFP(RNAi) clone was accomplished by cloning an ~1200 bp
amplicon (GFP_RNAi_02F: 5’- GACTCTAGAGGATCCCCGGGA -3’,
GFP_RNAi_02R: 5’- GAGCCTCAAACCCAAAACC TTC -3’) amplified from a bacterial
clone harboring pPD95.75, a plasmid containing a full-length copy of GFP (Fire kit 1995, Carnegie Institution of Washington, USA), into the Kpn-1 site of pPD129.36 (L4440) (Fire kit 1995). Ligated products were then transformed into 50 uL chemically competent HT115(DE3) cells by heat shocking the cells in the presence of 5 uL of ligation product. These *Escherichia coli* cells were then plated on LB + Ampicillin (100 ug/uL) and incubated at 37°C overnight. Transformant colonies were replica plated onto LB + AMP plates and PCR screened for the presence of the GFP amplicon using a T7 primer (5' - TAA TAC GAC TCA CTA TAG GGA GA- 3').

2.2. *Characterization of STARS-like proteins, T04A8.4 and F36F2.1*

2.2.1 *Experimental Procedure*

2.2.1.1 *Strains used*

For the following experiments, any comparisons between strains involved N2 and T04A8.4 (gk355) worms.

2.2.1.2 *Phalloidin-staining*

Phalloidin staining was performed as described by M. Koelle (2002, protocols section) with slight modifications. To examine the distribution of actin bundles in N2 and T04A8.4 (gk355), mixed stage worms were rinsed from a 150x15mm enriched NGM plate and washed 3x in M9 + 0.01 % Triton-X. Ten uL of compacted worms were flash frozen in liquid nitrogen and then immediately lyophilized in a speed-vac for 5 minutes. After drying, worms were incubated for 5 minutes in ~500 uL ice-cold acetone. The acetone was then siphoned-off by vacuum filtration and all residual acetone was removed.
by spinning in the speed-vac for 5 minutes. Next, 6 units of activated rhodamine-phalloidin (Invitrogen) was added to the tube containing the lyophilized worms. Twenty microliters of S mix (H$_2$O, 0.8 M Na$_2$PO$_4$ pH 7.5, 1 % SDS, 1 M MgCl$_2$) was added to the tube to dissolve the phalloidin. Worms were incubated in staining solution for approximately 2 hours, and then washed twice in PBBT (PBS containing 0.5 % bovine serum albumin and 0.5 % Tween-20). For viewing, stained worms were suspended in 20 uL mounting solution (90 % glycerol, 10 % PBS, 1 mg/mL phenylenediamine) and mounted on a slide for examination under a Zeiss Axiophot compound fluorescent microscope. Digital photos were taken using a Qimaging QICAM digital camera running Qcapture version 1.68.4 under 400x magnification.

2.2.2 RNAi construct design

Because previous RNAi screens had failed to resolve a phenotype for either T04A8.4 or F36F2.1 I designed new RNAi constructs to target the region of greatest homology between the two genes. Constructs were initially developed as described by Kamath & Ahringer (2003), however, a second set of constructs were developed using directional cloning techniques. A genomic fragment for each of T04A8.4 and F36F2.1 was amplified from N2 DNA by PCR using the Expand High Fidelity system (Roche) and primers with Kpn1 and Spe1 sites added. The amplicons were each cut with the appropriate restriction enzymes and cloned into pPD129.36 (provided by A. Fire) using T4 DNA ligase (Roche) and transformed into competent subcloning-efficiency DH5α (Invitrogen). Colonies that grew on LB + 100 ug/mL ampicillin plates were screened using a T7 primer to confirm the presence of an insert in the plasmid. Positive clones
were miniprepped using a Qiagen miniprep kit. One uL of plasmid DNA was then transformed into competent HT115(DE3). Positive colonies were once again screened with T7 primers to confirm the presence of an insert in the vector. Feeding of bacterial clones with RNAi constructs was performed as described above except in the case when a double-knockdown was attempted. In that case, overnight bacterial cultures for T04A8.4(RNAi) and F36F2.1(RNAi) were mixed (50/50) prior to plating on RNAi plates.

2.2.3 Construction of full-length translational fusions for microinjection

The T04A8.4 full-length construct was created by cloning a 4.4 kb PCR fragment into PstI and SmaI sites of the pPD95.75 vector (provided by A. Fire), which contains the gene that encodes GFP along with the 5’ untranslated region (UTR) of unc-54. The 4.4 kb fragment contains the entire T04A8.4 open reading frame (ORF) as well as 2.5 kb of sequence upstream of the gene. Primers were specially designed with specific restriction sites intended to eliminate the stop codon of the gene by replacing it with a glycine residue (GGG). Primer design also ensured that insertion of the genomic amplicon into the vector’s multiple cloning site resulted in T04A8.4 being in frame with GFP. Similarly, a translational fusion clone was constructed for F36F2.1 by amplifying a 3.2 kb fragment from genomic DNA and cloning it into pPD95.75. This amplicon contained the entire coding sequence for F36F2.1 plus ~1 kb of upstream region (Table 1).
2.2.3.1 Microinjection of C. elegans

T04A8.4 plasmid DNA (5 ng/uL) was coinjected with pRF4 *rol-6(su1006dm)* plasmid DNA (95 ng/uL) into the syncytial gonad of both N2 and T04A8.4(gk355) worms as described by Mello & Fire (1995). For F36F2.1, plasmid DNA was injected in a similar fashion, except that a concentration of both F36F2.1 and pRF4 plasmid DNA was 50 ng/uL.

2.3. Synthetic Genetic Interaction Matrix for T04A8.4 and F36F2.1

2.3.1 Strains used

The following mutant strains were used in the synthetic genetic interaction assay: N2, T04A8.4(gk355), *unc-120(st364ts), unc-45(e286), mua-6(rh85), unc-22(e66), unc-89(e1460), unc-98(su130), unc-95(su33), hlh-1(cc561), unc-97(su110), unc-87(e1216), cdc-42(ok825), cpn-3(gk336).*

2.3.2 Construction of the Synthetic Matrix

To assess the frequency of background embryonic lethality, each query strain was grown on RNAi plates seeded with HT115(DE3) bacteria containing L4440 with no insert. In brief, one L4 worm was cloned to an L4440(RNAi) plate and allowed to lay eggs overnight. Following 18 hours of egg laying these P₀ worms were removed from the plate. Embryos were given ~24 hours to hatch at which time embryonic lethality was scored. Similarly, each query gene’s RNAi phenotypes were examined and background embryonic lethality scored. This was accomplished as described above except that N2 worms were placed on RNAi plates for each of the genes under investigation.
2.3.2.1 Synthetic Genetic Interaction matrix assay

Synchronized L1 T04A8.4(gk355) worms were plated onto RNAi plates corresponding to each of the query genes mentioned above. These worms were allowed to develop to L4/young adult on the RNAi plates at which time a worm from each plate was cloned onto a fresh RNAi plate (in duplicate). Cloned worms were allowed to lay eggs overnight (~18 hrs) before they were removed. The following day RNAi plates were scored for embryonic lethality. Note that no allele of F36F2.1 was available, thus this assay was not carried out for this gene.

The reciprocal RNAi assay was carried out in which the query strains were each individually tested on T04A8.4 and F36F2.1. Embryonic lethality and post-embryonic phenotypes were scored as described above.
CHAPTER III

RESULTS

3.1. Muscle RNAi Screen

Given the difficulty of obtaining novel muscle-affecting genes by conventional mutagenesis approaches and the labor required to clone and map the locus, we set out to identify new muscle-affecting genes using RNA interference (RNAi). To confirm the validity of our approach we prepared RNAi constructs of the known muscle-affecting genes unc-97 (Gift from Guy Benian, Department of Pathology and Cell Biology, Emory University) and unc-54 and examined their effect on myofilament integrity. We fed bacteria expressing these constructs to myo-3::GFP worms and compared GFP localization to negative controls (no insert & no vector controls) and confirmed our approach was feasible (Fig. 5). The total number of worms displaying aberrant GFP localization was counted. Twenty-seven point four percent of worms fed control bacteria containing pL4440 displayed some form of myosin defect whereas 60.4% of worms fed unc-97(RNAi) had some form of myosin irregularity (no values were obtained for unc-54). In addition, we developed a positive control that targeted GFP. Because RW1596 worms depend on myo-3::GFP for viability beyond the two-fold stage of embryogenesis, GFP(RNAi) is lethal to most worms because it disrupts muscle myosin. Thus, feeding of the GFP(RNAi) construct to these worms resulted in embryonic lethality in many embryos. Although some animals persisted beyond embryogenesis they were usually quite sick, exhibiting uncoordinated to paralyzed movement, body morphology defects
Fig. 5. RNAi screen controls. Positive and negative controls were created to test the feasibility of this approach. (A) GFP(RNAi) results in embryonic lethality in a majority of embryos and severe myofilament disruption in surviving worms. Left panel is confocal fluorescence microscopy. Right panel is an overlay of fluorescence and DIC microscopy. (B) A second positive control used was *unc-97*, a component of muscle adherens junctions required for focal adhesion-like development. Left panel is confocal fluorescence microscopy. Right panel is an overlay of fluorescence and DIC microscopy. (C) No vector procedural control. This control confirmed that changing the diet of the worms from *E. coli* strain OP50 to *E. coli* strain HT115(DE3) has no effect on myofilament integrity. Left panel is an overlay of fluorescence and DIC microscopy. Right panel is confocal fluorescence microscopy. (D) The standard negative control used in this study was a no-insert control. HT115(DE3) bacteria, containing L4440 lacking a genomic insert between the two T7 promoters, were administered to RW1596 worms. Left panel is an overlay of fluorescence and DIC microscopy. Right panel is confocal fluorescence microscopy. Worms fed these bacteria were unaffected. Images captured using confocal microscopy. Scale bar= 50 um.
and slow growth. Microscopic examination of the myofilaments revealed dim GFP expression accompanied by severe disorganization of the myofilaments in 72.8% of all worms scored (Fig. 5A).

3.1.1 Age-related decline in sarcomeric integrity

It had been previously shown that RW1596 animals experience an acute loss of myofilament stability with age (Herndon et al., 2002). We found that L4/young adult worms, with 27.4% of screened animals displaying some myosin defects, are less likely to have myofilament abnormalities than animals scored 24 hours later (31.9%), 48 hours later (42.5%) or 72 hours later (75%) (Fig. 6).

3.1.2 The Muscle expressome-wide screen for gross RNAi phenotypes

To narrow and refine the search for novel muscle-affecting genes, we took advantage of the extensive amounts of expression data available for C. elegans muscle (McKay et al., 2004; David Miller, unpublished). We were able to compile a high confidence list of 3395 non-ribosomal/non-mitochondrial genes expressed in muscle. We screened this comprehensive list of muscle genes using targeted RNA-knockdown and detected gross (visible) RNAi phenotypes elicited by 717 (21.1%) different dsRNA’s. Of these, we identified phenotypes for 368 genes where no previous RNAi phenotype had been reported (Kamath et al. (2003; Simmer et al., 2003). Reciprocally, we failed to detect an RNAi phenotype for 349 genes that either Simmer et al. (2003) or Kamath et al. (2003) had previously described. Finally, 296/3395 clones were found to elicit defects in myosin localization in the
Fig. 6. Age-related decline in sarcomere integrity in *C. elegans*. The percentage of animals displaying myofilament defects (mutant) was calculated for each age group.
preliminary round of screening. Of these putative muscle-affecting genes, 91 (30.7%) exhibited an overt, visible RNAi phenotype.

The 296 clones that were found to elicit defects in myosin localization were retested both for overt phenotypic anomalies and for myofilament defects. Fifty-four clones had a visible, gross phenotype in the preliminary screen but not the rescreen, whereas 47 had an overt phenotype in the retesting phase but not in the initial screen. Twenty-eight clones were found to have gross RNAi phenotypes in both rounds of screening. Combining both rounds of screening, 129 of 296 (43.6%) clones gave both a myofilament phenotype and a gross anatomical RNAi phenotype.

The 296 dsRNAs found to elicit defects in myofilament integrity were characterized and the severity of the disruptions ranked using a penetrance scale. These classes are Wildtype class (WT), low class (LC), intermediate class (IC), and high class (HC), with the proportion of affected animals per scored plate being [0-25%), [25-50%), [50-75%) and [75-100%] respectively.

Of the muscle-affecting genes identified in the preliminary muscle screen, 45 (1.3% of all screened genes) fell into a high penetrance class and 251 (7.4% of screened genes) were classified into an intermediate penetrance class. Of the remaining clones examined, 1710 (50.3%) exhibited low penetrance defects while the remaining 1251 (37%) genes had no discernable myofilament phenotype (Fig. 7).

A secondary screen (rescreen) was performed on the 296 genes identified in the initial screen to reduce the likelihood of false-positives. The objective of the rescreen was to increase the total number of worms screened for each gene and to illustrate that the results obtained in the preliminary screen could be replicated. It is important to note that
Fig. 7. Preliminary RNAi screen results. A graphical depiction of the percentage of clones falling into each of the penetrance classes in the preliminary round of screening. A total of 1251 animals were designated wildtype (WT), 1710 low class (LC), 251 intermediate class (IC), and 45 were high class (HC).
the scoring stringency was elevated in the rescreen to help prevent false-positives from being flagged. Animals with only minor myofilament defects were counted as wildtype, whereas in the preliminary screen they were regarded as affected animals. The number of genes that rescreened positive in the more stringent secondary screen was 86. The total number of genes that retested negative, but whose sum remained either within the HC or IC classes, was 35. One hundred and sixty genes retested negative and fell below the threshold 50% value. It should be noted that of the 160 genes that fell below the cut-off, 43 had 45%, or greater, of all screened worms displaying myofilament defects (Fig. 8). All the aforementioned data on RNAi phenotypes and myofilament defects is publicly available at the muscle RNAi database (mRNAiDb):

http://www.zoology.ubc.ca/~alorch/rnai/.

Each clone, whose sum total of affected animals was greater than 50% after both rounds of screening, was individually screened by PCR to confirm that the clone contained the correct insert. A total of 139 genes were screened by PCR. Of these, 20 GenePair® primer sets failed to amplify a product. Clones for each failed PCR reaction were sequenced and aligned with the correct sequence. Two genes contained the correct insert, one gene had lost the genomic insert and 17 contained the incorrect insert. The clones containing the incorrect insert were misidentified, likely due to improper colony transfer, except in one instance. One clone contained an insert for a gene that is not available in the Ahringer library.
Fig. 8. Results of RNAi rescreen of putative muscle-affecting genes. A graphical depiction of the percentage of clones falling into each of the penetrance classes in the rescreen round of screening. A total of 2 clones fell into the WT penetrance class and 158 fell into the LC. A total of 121 clones successfully rescreened. Of these, 110 were designated as IC, and 11 were HC.
3.1.2.1 An online database of RNAi observations:

In order to handle the large amount of data produced during this expressome-wide screen, we have created a searchable database (constructed in collaboration with Adam Lorch) (Fig. 9) to store and index the information. The database (available at http://nrrr.ca/rnai) constructed for this screen is an online resource for the worm community to navigate and view the comprehensive list of identified muscle-affecting genes. Visitors to the muscle RNAi database (mRNAiDb) will be able to search the database by gene, cosmid, well-position or a key word (i.e. helicase, kinase, LIM domain etc). In addition, the mRNAiDb allows users to search for specific phenotypes with or to the exclusion of other phenotypes (i.e. Pat and Unc or Pat and not Ste). The database contains the results obtained for each gene screened including gross RNAi phenotype, myofilament anatomy, presence/absence of human homolog as determined by INPARANOID (Remm et al. 2001) and the presence/absence of transmembrane domains as determined by PHOBIUS (Kall et al. 2004). The program classifies each gene as either ‘muscle-affecting’ or normal depending on the number of defective animals observed in the screen of that particular gene. In addition, images of each putative muscle-affecting gene will be available.

3.2. Screening for novel muscle-affecting genes reveals a pair of paralogs, T04A8.4 and F36F2.1, of unknown function that are required for proper myo-3::GFP localization

In our screen for muscle-affecting genes we uncovered a pair of paralogous proteins that exhibit defective myosin localization when treated with their respective dsRNA’s (Fig. 10). The genes encoding these proteins, T04A8.4 and F36F2.1, share
Fig. 9. The mRNAiDb (http://nrrr.ca/rnai). We have constructed a database to store and manage the information obtained in this screen. This online source allows users to query the database for any cosmid, gene, RNAi phenotype or description. As well, the scoring results for all clones can be retrieved. The mRNAiDb was constructed in collaboration with Adam Lorch (computer technician, Moerman lab, University of British Columbia).
Fig. 10. RNA-mediated interference of T04A8.4 and F36F2.1. In (A), T04A8.4(RNAi) results in animals that display disorganized and discontinuous myofilaments. In (B), F36F2.1(RNAi) administered animals display large deposits of myo-3::GFP within the filaments. (C) No insert control (RNAi) The bacterial clones fed to these worms contain L4440 without an inserted genomic fragment. All worms are L4/young adult stage. All images captured using a Zeiss Axiophot compound fluorescent microscope. Scale bar= 50um.
significant regions of homology at their C-termini (BLAST score = 7e-23). Feeding T04A8.4(RNAi) bacterial clones to myo-3::GFP animals results in large deposits of myosin, often accompanied by disorganization of the filaments and gaps that interrupt parallel fibres. The T04A8.4 clone initially fell into the IC phenotypic class, but failed to rescreen. A comparable myofilament phenotype was also observed in F36F2.1(RNAi) treated animals. This gene initially screened positive for sarcomeric irregularities, but a reexamination of this gene resulted in its drop to the LC phenotypic class.

3.2.1 T04A8.4 is required for proper actin localization

Rhodamine-phalloidin staining revealed significant defects in actin localization (Fig. 11A) and reduced staining intensity in T04A8.4(gk355) worms when compared to N2 worms (Fig. 11B). These defects include interruptions in the longitudinally oriented actin bundles in body wall muscle and a general disorganization of the microfilaments.

3.2.2 Expression data

GFP promoter fusions (courtesy of D. Baillie, Dept. of Molecular Biology and Biochemistry, Simon Fraser University) show that T04A8.4 is expressed primarily in body wall muscle and the terminal bulb of the pharynx (Fig. 12A). The other STARS homolog, F36F2.1, also appears to be expressed in the terminal bulb of the pharynx (Fig. 12B), but only very weakly in body wall muscle (data not shown).

An examination of SAGE data showed that a single transcript of T04A8.4 was detected in the SW031 SAGE library (derived from FACS-sorted muscle cells-replicate 2). Transcripts were also detected in SAGE libraries for ciliated neurons, the gut and glp-
Fig. 11. Rhodamine-phalloidin staining of young adult worms. In (A), T04A8.4(gk355) worms (left) are adjacent to N2 worms (right). Notice the reduced staining intensity in the T04A8.4(gk355) worms compared to the N2 worms. As well, fracturing of the laterally-oriented actin filaments is evident in T04A8.4(gk355) when compared to N2 worms. Arrowheads indicate the position of the fractures. Image in A is magnified 400x. In (B), N2 worms (left) have intense staining in the vulva (indicated by arrowheads) and the longitudinally oriented bundles of actin relative to the T04A8.4(gk355) (right) Images are magnified 200x in B. Scale bar= 50um.
Fig. 12A. Promoter GFP fusion. Expression of a transcriptional-fusion reporter construct containing the promoter of T04A8.4 fused to GFP. Green fluorescence is localized to the body wall muscle. (A) Confocal image of an adult hermaphrodite worm expressing the reporter construct. (B) Nomarski image of the same adult hermaphrodite. Arrow head indicates region of coexpression of the STARS paralogs. Scale bar= 50um.

Fig. 12B. Promoter GFP fusion. Expression of a transcriptional-fusion reporter construct containing the promoter of F36F2.1 fused to GFP. Green fluorescence is localized to the terminal bulb and corpus of the pharynx. (A) Confocal image of an adult hermaphrodite worm expressing the reporter construct. (B) Nomarski image of the same adult hermaphrodite. Arrow head indicates region of coexpression of the STARS paralogs. Scale bar= 50um.

Fig. 12C. T04A8.4 is expressed in the pharynx. (A) Faint GFP expression can be detected in the pharynx. Diffuse expression also present in body wall muscle and possibly the spermatheca. (B) A schematic diagram of the translational fusion between T04A8.4 and GFP. The construct contains 2.5 kb upstream of the gene. Scale bar= 50um.
4 adults. F36F2.1 tags were detected in the SWEM1 (FACS-sorted muscle, long-SAGE) (1 tag) and SW031 (FACS-sorted muscle cells, replicate 2) (11 tags) libraries.

Full-length GFP translational fusions were constructed for both T04A8.4 and F36F2.1 (as well as other muscle genes identified in the RNAi screen: C46F4.2, F28H1.2, T22B2.4) and attempts were made to coinject the clones with pRF4 (a vector containing a dominant marker which causes rolling of transformed worms) into both N2 and T04A8.4(gk355) worms (see Table 2). Roller worms were obtained when pNAD2 (T04A8.4::GFP) was microinjected into N2 worms, however these worms were sick and uncoordinated, and died before progeny were laid. Because of the possibility that overexpression of T04A8.4 is lethal to worms, I tried injecting T04A8.4(gk355) worms with the pNAD2 construct. F1 roller worms were obtained, however no GFP fluorescence could be observed. F2 rollers were obtained (n=4) and examined for GFP signal. Fluorescence was observed in the pharynx of roller worms (Fig. 12C). Similarly, F36F2.1::GFP (pNAD9) was injected into N2 worms, but only non-GFP, roller progeny were obtained.

3.2.3 Synthetic genetic interactions

Mutational analysis of Saccromyces cerevesiae and C. elegans has shown that the majority of genes within an organism are not essential (Tong et al., 2001). Animals can tolerate loss-of-function in many of these non-essential genes with no apparent effect on viability, development or movement, a phenomenon known as phenotypic robustness (Gu et al., 2003; Conant et al., 2004; Hunter et al., 2005). Synthetic genetic interactions between proteins in similar or redundant pathways have been suggested as the
mechanism behind phenotypic robustness. A synthetic genetic interaction matrix was
constructed to test for interactions involving T04A8.4 with other known muscle-affecting
Table 2. A list of functional fusions that were constructed to validate results from the RNAi screen. Primers for functional fusions were designed to target the entire open reading frame for the gene plus a specified region upstream of the gene. The amplicon was then ligated into a GFP expression vector and the complete plasmid coinjected into young adult worms with a *rol-6* marker. Constructs were then sequenced to confirm the presence of the insert and that the amplicon was in frame with the GFP.

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genes. To test the feasibility of this approach, I performed a reciprocal RNAi-mutant analysis for unc-120 and hlh-1, two genes previously shown to form a synthetic genetic interaction (Hunter et al., 2005). I was able to show that unc-120(RNAi); hlh-1, but not its reciprocal hlh-1(RNAi); unc-120, results in enhanced embryonic lethality (40% and 0%, respectively). Although the reciprocal genetic interaction between unc-120 and hlh-1 was not detected, the fact that we did observe a highly significant increase in lethality in one interface suggests that this approach is a viable means of identifying genetic interactions.

Synthetic genetic analysis of the known muscle-affecting genes against the query gene T04A8.4 identified several possible interactions. One potential lethal interaction between T04A8.4 and unc-98 was found (Fig. 13), as well as a synthetic enhancement interaction between unc-22 and T04A8.4 (Fig. 14). Disruption of function by mutation or RNAi of any of these genes alone did not result in embryonic lethality or sterility. However, T04A8.4(gk355); unc-98(RNAi) resulted in 16.2% embryonic lethality. The reciprocal interaction, unc-98(su130); T04A8.4(RNAi) resulted in completely sterile animals. Similarly, both interactions tested for unc-22 with T04A8.4, unc-22; T04A8.4(RNAi) and T04A8.4(gk355); unc-22(RNAi), resulted in either reduced fecundity or total sterility.

F36F2.1(RNAi) was administered to each of the known muscle genes under investigation. It was found that a genetic interaction likely exists between F36F2.1 and unc-87, and another interaction with cdc-42 (Fig. 13, 14). The reciprocal interaction could not be tested given that no allele of F36F2.1 was available. F36F2.1(RNAi); cdc-42(ok825) animals are completely sterile unlike cdc-42(ok825) animals on control
pL4440 plates which have normal fecundity. Feeding F36F2.1 dsRNA to *unc-87* animals results in a reduction of brood size and lethality in 56% of all embryos.
Fig. 13. Synthetic lethal screen. Two query genes, T04A8.4 and F36F2.1, were tested for synthetic genetic interactions with a series of non-essential known muscle-affecting genes. Each gene was scored for embryonic lethality and percentage of dead embryos was calculated. The color coded box indicates the percentage of dead embryos for the given gene. The heat matrix is assembled such that the RNAi construct is on the X axis and the mutant allele of the gene is on the Y axis.
RNAi (Y)/mutant (X)   | N2 | T04 | F28 | unc-45 | unc-120 | mua-6 | unc-22 | unc-89 | unc-98 | unc-95 | hlh-1 | unc-97 | cdc-42 | unc-87 | atn-1 |
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Fig. 14. Synthetic enhancement screen. Two query genes, T04A8.4 and F36F2.1, were tested for synthetic genetic interactions with a series of non-essential known muscle-affecting genes. Each mutant strain (X axis) was fed bacteria expressing dsRNA corresponding to each gene on the Y axis and scored for brood size. If a strain had fewer than 25 progeny it was designated as a small brood size (SBS). If there were fewer than 5 progeny for a strain it was called sterile (STE).
CHAPTER IV

DISCUSSION

In C. elegans, the introduction of foreign dsRNA triggers a cellular response that culminates in the abatement of endogenous mRNA complementary to the invading dsRNA (Timmons and Fire, 1998; reviewed in Fire, 1999). This convenient attribute can be exploited by researchers to directly target a given gene for knockdown, thus allowing for the examination of the loss-of-function phenotype of that gene. We used an RNAi feeding library (Kamath & Ahringer, 2003) to screen all non-ribosomal/non-mitochondrial genes expressed in body wall muscle to identify novel muscle-affecting genes (Fig. 3). The source of this list of genes, which we refer to as the muscle expressome, was derived from both SAGE (McKay et al., 2003) and DNA microarray (R. Fox and D. Miller, unpublished) experiments on purified embryonic muscle cells. We fed bacteria expressing dsRNA corresponding to each gene in the muscle expressome to C. elegans strain RW1596, a strain harboring a rescuing extrachromosomal array of myo-3::GFP. This allowed for straightforward visualization of the myofilaments under a compound fluorescent microscope.

4.1 RNAi screening of the muscle expressome yields a variety of putative muscle-affecting genes:

We have screened 3395 genes using a myo-3::GFP reporter for abnormalities in sarcomere integrity. The myofilament distortions we have observed range from small aggregates of GFP to large deposits, which may be within the filament or adjacent to it.
We also see gradients in disorganization of the myofilaments ranging from mild discontinuities in the fibers to large interruptions often accompanied by accumulation of the GFP reporter protein (Fig. 4). These defects may be the result of several factors, including abnormal or aberrant localization of myosin heavy chain, early onset sarcopenia, loss of sarcomere stability and/or defective myosin assembly. It is also possible that these defects result from loss of proteins within muscle (i.e. sarcomere anchoring proteins) or from the loss of proteins that secure the sarcomere to other tissues (i.e. hemidesmosomal proteins). Although the approach we used to identify novel muscle-affecting genes is quite sensitive, it is not able to identify the root cause of the myofilament abnormalities. More research is required to determine the cause of each of the defects we observed.

In addition to the myofilament screen, we scored all clones for their ability to confer overt RNAi phenotypes on C. elegans strain RW1596 (see Table 1 for a list of all phenotypes scored). We observed 717 (21%) clones with overt phenotypes out of 3395 genes screened. Of these, 149 genes were found to be indispensable for viability, 170 displayed uncoordinated movement defects and 19 genes exhibited Pat phenotypes. Of the 717 visible phenotypes observed in the RNAi screen, 349 had been previously shown to have a phenotype by either Simmer et al. (2003) or Kamath et al. (2003). Thus, we have defined new RNAi phenotypes for 368 genes. Reciprocally, we failed to observe an RNAi phenotype for 349 genes that either Simmer et al or Kamath et al. had previously shown to display a phenotype. This observed discrepancy may result from several factors, but the most likely in this case is that we were using a hypersensitive strain of C. elegans.
The genetic background of *C. elegans* RW1596 may have had the effect of making RNAi-treated worms more sensitive to insults targeted to muscle. This does not imply that *C. elegans* strain RW1596 is more susceptible to RNAi, rather concurrent knockdown of two muscle genes, in this instance *myo-3* and any query gene under investigation, results in a stronger, more penetrant phenotype than would otherwise be observed. In addition to a change in the closely controlled levels of myosin A, a barrel-shaped GFP reporter has been fused to *myo-3*, likely preventing the tight packing of available myosin A molecules in the central bipolar H zone of thick filaments. This would negatively affect the levels of myosin B and paramyosin, as well as prevent the proper assembly of these molecules into a functional thick filament (White et al., 2003). Thus, it is very likely that GFP-tagging of MYO-3 results in looser packing of the myosin rods, thereby making the strain more sensitive to RNAi treatment with clones of other muscle genes.

As previously mentioned, we failed to identify phenotypes for 349 genes that either Simmer et al. (2003) or Kamath et al. (2003) had uncovered in their genome-wide RNAi screens. This is likely a consequence of our screening methodology. Overt phenotypic scoring was performed at a specific time in the development of the worms. Rather than scoring for RNAi-induced phenotypes at all developmental stages, we only scored L4/young adult worms. Thus, our screen was not able to identify late onset RNAi phenotypes such as egg-laying defects or adult lethality. In addition, several clones that had been demonstrated to induce locomotory defects were not identified likely the result of late onset penetrance.
It became evident early in the screen that *C. elegans* strain RW1596 animals suffer a gradual loss of myofilament integrity as they mature. This phenomenon, known as sarcopenia, was first described by Herndon et al. (2002) using this identical strain. The authors found considerable variability in both the time of onset and rate of decline within an isogenic population maintained under identical conditions, which suggests that age-related sarcopenia has a significant stochastic component. We found that sarcopenia is less pronounced in L4/young adult animals and therefore chose to screen this cohort only (Fig. 15).

The stochastic nature of the age-related decline in sarcomeric integrity combined with intrinsic variability in RNAi penetrance led us to devise a classification system for the myofilament-integrity screen. In this system we catalog genes into four phenotypic classes based upon the number of individuals on a slide exhibiting some sort of myofilament defect: The wildtype class (WT), the low class (LC), the intermediate class (IC) and the high class (HC). The WT and LC classes represent genes that we believe do not affect the integrity of the sarcomere, as they are not significantly different from control-fed worms. Genes whose dsRNA elicited defects in greater than 50% (the IC and HC classes) of scored worms were classified as muscle-affecting genes. Thus, for the purposes of this thesis we will refer to genes in the IC and HC as putative muscle-affecting genes and genes within the LC and WT classes as wildtype or normal.

4.1.1 The preliminary RNAi screen

A comprehensive list of genes expressed in embryonic muscle by both SAGE and DNA microarrays (McKay et al., 2004; David Miller, unpublished) was compiled in
Fig. 15. Age-related sarcopenia in *C. elegans*. RW1596 worms undergo a gradual loss of myofilament integrity as they age. Although a small percentage of all worms examined had a range of defects, the fewest disruptions are observed in L4 and young adult worms. Scale bar= 50um
order to focus our screen on genes that function within nematode body wall muscle. This muscle expressome-wide screen was divided into two parts. The first component of the screen was the preliminary round of screening where each gene was tested for RNAi-mediated defects in myo-3 localization within muscle cells. This approach yielded a windfall of potentially novel ‘muscle-affecting’ genes. We believe that the high percentage, 8.7%, of genes affecting sarcomere stability is the result of enriching for genes in muscle prior to screening. Additionally, animals with minor myofilament irregularities were scored as ‘defective’, which may have contributed to an artificially high number of putative muscle-affecting genes. We classified 296 genes as either high penetrance (HC) or intermediate penetrance (IC) in the first round of screening (see Fig. 7). A large proportion of the genes that fell into the HC class have no known function. Of the 45 HC genes that were identified in the preliminary screen, 19 have human orthologs according to INPARANOID algorithms (Remm et al. 2001). This is significant because it provides researchers studying nematode and mammalian muscle with a pool of genes possibly harboring new functions affecting sarcomere stability and integrity. In addition, 11 of the 45 HC genes have predicted transmembrane domains (Kall et al. 2004), with 5 transmembrane positive genes also having human orthologs. A transmembrane domain could indicate some role for the protein in anchoring the sarcomere to the sarcolemma. Clearly, these genes offer significant promise as candidates for further study.

Many other genes were identified as putative muscle-affecting genes in the first round of screening. Of the 296 genes identified as candidates, 251 fall into the IC class. A total of 15/21 known muscle-affecting genes screened in this RNAi study were successfully identified, indicating that this approach captures close to 70% of all known
genes that affect sarcomere integrity. Several known muscle-affecting genes flagged in this screen provided validity to our approach. For example, we have found defects in the muscle proteins *unc-15* (paramyosin), *unc-95* and *vab-10*, as well as several others (see Table 3). Interestingly, worms fed *unc-15* dsRNA appeared phenotypically normal both in our preliminary screen and in the genome-wide screen of Simmer et al. (2003), but upon close examination of the myofilaments it was quite evident that abatement of paramyosin mRNA resulted in defective myosin localization. This is significant given that paramyosin forms the core region of the filament around which myosin molecules assemble and thus you would expect that decreased paramyosin protein should affect the stability of the myofilaments. *vab-10*(RNAi) also produced significant defects in GFP localization (Fig. 16). *vab-10* is required for the mechanical pliability of the epidermis in response to stress caused by contraction of actin filaments and for elongation during embryogenesis. Bosher et al. (2003) demonstrated that *vab-10* had two isoforms, A and B, that are both expressed primarily in the epidermis, but that VAB-10B is also expressed in muscle. The authors noticed that the epidermis detached from the cuticle apically and muscle basally in *vab-10*(RNAi) worms. Similarly, we observe attachment defects when feeding *vab-10* dsRNA, but we also detect severe faults in myofilament structure and loss of GFP signal in these worms. This implies that VAB-10 may function not only to maintain the integrity of the fibrous organelles, but also to preserve the stability of the sarcomere. Perhaps the connection between VAB-10/Spectroplakin and some muscle cell component is required to keep the myofilaments in register. It is also possible that VAB-10B is functioning within muscle to structure the sarcomere. Alternatively, it is also
Fig. 16. RNA-mediated interference of *vab-10* results in severe muscle defects. *vab-10*(RNAi) results in a severe muscle phenotype characterized by large breaks in the myofilaments and loss of entire regions of *myo-3::GFP* expression. Worms fed *vab-10* dsRNA are shown in A (100x magnification) and B (200x magnification). Scale bar= 50um
Table 3. A list of the known muscle-affecting genes that were successfully identified in this RNAi screen. A total of 15/22 (68.2%) of known-muscle affecting genes were identified in the screen.

<table>
<thead>
<tr>
<th>Cosmid/gene</th>
<th>Function/ G.O</th>
<th>Cellular location</th>
<th>RNAi gross phenotype</th>
<th>Phenotypic class (sum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-89</td>
<td>protein kinase</td>
<td>M line</td>
<td>unc</td>
<td>HC</td>
</tr>
<tr>
<td>unc-15</td>
<td>paramyosin homolog</td>
<td>A band</td>
<td>WT</td>
<td>HC</td>
</tr>
<tr>
<td>vab-10</td>
<td>Spectraplakin</td>
<td>epidermal cells</td>
<td>emb, gro, ste</td>
<td>HC</td>
</tr>
<tr>
<td>unc-95</td>
<td>LIM domain</td>
<td>dense body</td>
<td>unc</td>
<td>HC</td>
</tr>
<tr>
<td>pat-4</td>
<td>integrin-linked kinase</td>
<td>dense body</td>
<td>WT</td>
<td>HC</td>
</tr>
<tr>
<td>unc-45</td>
<td>myosin assembly protein</td>
<td>A band</td>
<td>WT</td>
<td>IC</td>
</tr>
<tr>
<td>mup-4</td>
<td>Fibrillin</td>
<td>membrane</td>
<td>WT</td>
<td>IC</td>
</tr>
<tr>
<td>pat-6</td>
<td>alpha-parvin</td>
<td>N/A</td>
<td>WT</td>
<td>IC</td>
</tr>
<tr>
<td>unc-105</td>
<td>muscle degenerin</td>
<td>N/A</td>
<td>dpy</td>
<td>IC</td>
</tr>
<tr>
<td>myo-3</td>
<td>Myosin heavy chain A</td>
<td>sarcomere</td>
<td>SBS, unc</td>
<td>HC</td>
</tr>
<tr>
<td>unc-112</td>
<td>Required for assembly of muscle dense bodies and M lines</td>
<td>Dense body/M line</td>
<td>gro</td>
<td>IC</td>
</tr>
<tr>
<td>dim-1</td>
<td>Immunoglobulin and related proteins</td>
<td>dense body</td>
<td>slo</td>
<td>IC</td>
</tr>
<tr>
<td>uig-1</td>
<td>Putative guanine nucleotide exchange factor</td>
<td>dense body</td>
<td>WT</td>
<td>IC</td>
</tr>
<tr>
<td>unc-52</td>
<td>Perlecan</td>
<td>basal membrane</td>
<td>adl, prz, unc</td>
<td>IC</td>
</tr>
<tr>
<td>unc-23</td>
<td>Molecular chaperone</td>
<td>N/A</td>
<td>gro</td>
<td>IC</td>
</tr>
</tbody>
</table>
possible that loss of VAB-10 function triggers detachment of muscle from the hypodermis, resulting in a collapse of the myofilament lattice.

It should be noted that several genes previously shown to be required for proper muscle assembly/maintenance were not identified in the muscle RNAi screen (see Table 4). For example, RNAi treatment of animals with pat-3/β-integrin dsRNA, despite having displayed both sterility and uncoordinated movement, did not exhibit a highly penetrant disorganized myofilament phenotype, and therefore it was assigned to the LC penetrance class. As well, the sarcomeric protein UNC-98 did not display either an overt RNAi phenotype or a myofilament-defect phenotype. A total of 7 of 22 known muscle-affecting genes were missed by our screen, indicating a false-negative rate of 32% (Table 4). There are several possible explanations for not detecting these genes, including the inherent variability in RNAi screening, loss of genomic insert in the bacterial clone, stoichiometric effects of RNAi or the means by which we screened animals for myofilament defects.

RNA-mediated interference by feeding small interfering dsRNA’s to worms has been shown to display variable penetrance of the RNAi-effect (Timmons et al., 1998; Simmer et al., 2003). Simmer et al. (2003) observed significant differences between different laboratories and investigators and between experiments done within the same laboratory by the same investigators. Data sets obtained from Simmer et al. (2003) display only 75% concordance with Fraser et al. (2000), whereas Fraser et al. detected RNAi phenotypes for 84% of the clones for which Simmer et al. found a phenotype. Thus, experiments done in the same laboratory can vary from 10%-30%, a phenomenon that results almost exclusively from false-negatives (Simmer et al., 2003). Similarly, the results from our overt RNAi screening suggest a similar trend. The results of our screen
Table 4. Known muscle-affecting genes not identified in the RNAi screen. A total of 7/22 of known muscle-affecting genes were not flagged in the screen, suggesting that this screen had a false-negative rate of 31.8%.

<table>
<thead>
<tr>
<th>Cosmid/gene</th>
<th>Function/ G.O</th>
<th>Cellular location</th>
<th>RNAi gross phenotype</th>
<th>Phenotypic class (sum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-805</td>
<td>myotactin homolog</td>
<td>hypodermal</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pat-3</td>
<td>Integrin beta subunit</td>
<td>Dense body/M line</td>
<td>ste, unc</td>
<td>LC</td>
</tr>
<tr>
<td>spc-1</td>
<td>Ca2+-binding actin-bundling protein</td>
<td>basolateral region of membrane</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>atn-1</td>
<td>Ca2+-binding actin-bundling protein</td>
<td>dense body</td>
<td>slo</td>
<td>LC</td>
</tr>
<tr>
<td>unc-98</td>
<td>Zn-finger protein</td>
<td>dense body/M line</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>mlc-3</td>
<td>myosin essential light chain</td>
<td>sarcomere</td>
<td>gro</td>
<td>WT</td>
</tr>
<tr>
<td>hlh-1</td>
<td>MyoD homolog</td>
<td>Muscle progenitors</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>
showed a 49% rate of concordance with the results from Simmer et al. and Kamath et al. (2003), whereas the results of either of their screens gave 58% concordance when compared to our screen.

Another possible explanation for the high false-negative rate is that the clones that were being tested had lost the insert from the feeding vector. These clones would not produce dsRNA and thus no short-interfering RNA (siRNA) would be made to knock-down the endogenous mRNA. This can arise during the successive transfers of the bacterial colonies from glycerol stocks to media plates. We observed a loss-of-insert event for 1 gene out of 139 genes tested by PCR.

Since we limited our screening to L4/young adult worms, we may have inadvertently limited our ability to detect some RNAi phenotypes. For example, some phenotypes do not manifest themselves until later in development. This was an effect we observed with unc-54 (data not shown), where animals treated with unc-54 dsRNA became uncoordinated, and their myofilaments more disorganized, as adults.

4.1.1.1 Rescreen of putative muscle-affecting genes

After each of the genes within the muscle expressome had been scored for myofilament defects we rescreened all genes that fell into the IC and HC classes in the preliminary screen. Each of the 296 genes identified were reexamined for both gross (overt) phenotypic irregularities and sarcomere deficiencies. To reduce the likelihood of false-positives, we increased the stringency of our screening by simultaneously increasing the number of worms screened and by elevating the scoring stringency of the myofilament defects. In this round of screening, a clone was classified in three different
ways depending on the percentage of ‘affected’ animals observed during scoring. In the first instance, designated (+/+), was assigned to a gene that rescreened positive, and therefore had a net positive sum for both screens. The second type of clone encountered was a gene that rescreened negative, but the sum total of animals with myofilament defects was still greater than 50% (-/+). Finally, a gene that rescreened negative and whose sum total of worms exhibiting sarcomeric deficiencies was less than 50% was assigned to the (-/-). With these criteria, we have successfully identified 86 genes in the (+/+ class and another 35 clones were classified as (-+) for a total of 121 (3.6%) muscle-affecting genes. This double screen and high stringency yields a high degree of confidence list of muscle-affecting genes (see Fig. 8).

An examination of the types of genes (as determined by KOGs) identified in the RNAi screen reveals that a significant proportion, 49, had no annotated function or KOG domain (see Fig. 17); yet, almost 20% (9) of these unknown genes have close human orthologs (as determined by INPARANOID). Identifying unknown genes that function to maintain the integrity of the sarcomere is a clear example of the power of this RNAi screen. One small group of genes that offer significant promise is the unknown LIM domain-containing proteins (Fig. 17). We identified three uncharacterized LIM proteins in this screen, F25H5.1, C02F5.7 and F20D12.5. Given that LIM domain proteins have been implicated in several processes within body wall muscle, the identification of these novel proteins could prove to be quite significant. Other classes of genes that are highly represented within the muscle RNAi database (mRNAiDb) are metabolic (25) and signaling genes (13). Researchers interested in understanding how muscle stress and damage is
Fig. 17. Types of genes identified in the RNAi screen as determined by NCBI KOGs. Only the 121 genes that remained in either IC or HC after the rescreen were considered. Genes with human orthologs are represented by maroon bars. Those without are represented by blue bars.
transduced into new protein synthesis should be interested in examining the group of
genes isolated in this RNAi screen (see Fig 17).

4.1.1.2 Role of the proteasome in protein turnover in muscle

One salient feature observed on scanning the mRNAiDb is that RNAi of genes
involved in protein turnover have a strong tendency to cause defects in sarcomere
structure. Protein degradation by the ubiquitin/proteasome system is known to occur in
muscle cells, and defects in protein turnover have been implicated in some human
diseases, including Alzheimer’s and Parkinson’s disease (reviewed in Hol et al., 2005). In
C. elegans, UNC-45, a molecular chaperone for myosin, has been shown to be
multiubiquitylated by the E4 protein CHN-1 and UFD-2 (Barral et al., 2002; Hoppe et al.,
2004). This CHN-1/UFD-2-dependent multiubiquitylation of UNC-45 is postulated to
indispensable for proper myosin folding and assembly into thick filaments. Our screen
for novel muscle-affecting genes uncovered several genes which likely function in
protein degradation/turover. RNAi of this class of genes often resulted in severe muscle
defects. Thus, a closer examination of these genes may shed light on several questions
that have yet to be addressed in nematode muscle: how does muscle stress induce a
signalling cascade that ultimately leads to the renewal of sarcomeric and FA proteins?
What are the signalling components that are required to transduce sarcomere or FA
damage into new protein translation? Finally, how are the multiprotein complexes of
sarcomeres and FA’s, with all their intricate connections, turned-over and sent for
destruction? These are all avenues that I am interested in pursuing further.
4.1.1.3 The RNAi screen identifies T04A8.4 and F36F2.1, two homologs of the human
STARS protein:

Two paralogous genes, T04A8.4 and F36F2.1, identified in the RNAi screen for
defective sarcomeres are homologous to the human STARS protein. Most of this
sequence similarity occurs at the last 154 of 375 amino acids of the C-terminal end of the
mammalian STARS protein (T04A8.4 e value = 2.2e –19, F36F2.1 e value = 1.5e -28)
(Fig. 18). The C-terminal region of the STARS protein was shown to be necessary and
sufficient to bind and stabilize actin stress fibres in transfected COS cells (Arai et al.
2002). It has been demonstrated that mammalian STARS localizes to the I-band of
skeletal and cardiac tissue in humans where it has been proposed to promote serum
response factor (SRF) transcriptional activity through the binding of actin stress fibers
and an interaction with RhoA (Arai et al., 2002). Given the subcellular localization of
STARS and that it has been shown to be expressed during myofibrillogenesis, it was
suggested that this evolutionarily conserved protein may play a role in sarcomere
assembly through its interaction with actin (Arai et al., 2002).

I have provided evidence that the C. elegans STARS homolog T04A8.4 is
expressed in both body wall muscle and the terminal bulb of the pharynx and that its
paralog F36F2.1 is coexpressed in the pharynx (Fig. 12 A,B). Furthermore, I have shown
that RNAi of these genes results in an overtly wildtype phenotype with moderate defects
in myofilament anatomy (Fig. 10). These genes were identified in the preliminary round
of screening, but were not reconfirmed in the rescreen. Personal observations in separate
RNAi screens indicate that both T04A8.4 and F36F2.1 should fall into the IC penetrance
class (52.1% and 54.6%, respectively). This discrepancy between the two sets of results
Fig. 18. Alignment showing the region of greatest homology between the mammalian STARS protein and its two C. elegans orthologs T04A8.4 and F36F2.1. The alignment begins at amino acid number 154. Identical matches between amino acids are indicated by dark shading. Similarly charged amino acids are indicated by light shading. No shading indicates that no conservation exists at that position in the alignment.
may be the consequence of intrinsic variability of RNAi screening (Simmer et al. 2003) or it may mean that the defects we first observed were the result of stochastic age-related decline in myofilament integrity. Alternatively, the penetrance of the myosin-defect phenotype in these genes may be lower than the threshold value that we chose for our RNAi screen. This could be the case if these proteins were functioning (at least partially) as enzymes. Most enzymes function as catalysts, thus incomplete knockdown by RNAi would severely reduce the penetrance of any RNAi phenotype. Expression data (http://tock.bcgsc.ca/cgi-bin/sage140) indicate that both T04A8.4 and F36F2.1 are expressed in low levels within FACS-sorted embryonic muscle cells (McKay et al. 2004). Low levels of a given transcript could be indicative of two things: very little protein product is required to perform the function or it could indicate a disconnect between the observed message levels and number of translated transcripts. That is to say, a single message may be rapidly translated into several proteins. Thus, RNA-mediated knockdown of these transcripts may be an inefficient means to silence the genes. For instance, if the RNAi machinery fails to eliminate all transcripts for a rapidly translated message, the result would be either low penetrance defects or no observable phenotypes. Clearly, more work is required to determine if loss of either, or both, of these proteins results in true myofilament defects. This may be a difficult task if the penetrance of these defects is low because these irregularities can easily be confused with age-related sarcopenia.

Phalloidin staining of a putative null allele of T04A8.4, gk355, shows decreased staining intensity of actin microfilaments compared to N2 worms, as well as disorganization and fracturing of the laterally-oriented bundles of actin in body wall muscle cells (Fig. 11A,B). These defects do not impair locomotory function of the
worms, but it may be responsible for the significant increase in body length of T04A8.4(gk355) worms relative to N2 worms (Fig. 19).

Preliminary data from microinjections of full-length reporter fusions of T04A8.4 or F36F2.1 into N2 worms, under their endogenous promoters, suggests that overexpression of T04A8.4 or F36F2.1 proteins affect viability of the transformed animals. Several F1 roller worms (n=12) were obtained for T04A8.4::GFP, but all transformed worms were sick, failed to show GFP expression and eventually died. Other attempts to microinject full-length T04A8.4 into T04A8.4(gk355) worms did not produce GFP-expressing worms (Table 2). Roller F1 worms (n=15) were obtained after microinjecting F36F2.1::GFP, but only four F2 roller was obtained. These F2 roller worms had body morphology defects, uncoordinated movement and expressed GFP diffusely in the pharynx (fig. 12C). To confirm that these constructs were true translational fusions, all vectors were sequenced and aligned (using MultiAlin) to the annotated sequence data from Wormbase (www.wormbase.org). Most constructs had the correct insert in frame with GFP (see Table 1).

These data, although incomplete, indicate that the C. elegans homologs of STARS function in muscle acto-myosin dynamics. A question that needs to be addressed is how T04A8.4 and F36F2.1 are related. Given that they share significant homology we postulated that these two proteins might act redundantly to control actin-myosin localization in muscle, and therefore would be in-paralogs. To test this hypothesis, we fed RNAi constructs for both genes simultaneously to RW1596 worms. Simultaneous feeding of the two RNAi constructs did not intensify the myofilament defects observed when either construct was fed alone (data not shown). This may mean that these two
Fig. 19. Mean worm length for N2 and T04A8.4(gk355) worms. Young adult stage (4 day old) N2 worms (n=20) were compared to T04A8.4(gk355) (n=20) animals by measuring the worm from the tip of the tail to the end of the head (length measured in arbitrary units). It is evident that T04A8.4(gk355) worms are on average 31 arbitrary units larger, or ~9% larger. Data compiled by Andrew Giles (Graduate student, Rankin lab, University of British Columbia).
genes are out-paralogs and thus not true homologs. If this is the case these genes likely function independently in separate and distinct pathways to maintain the integrity of the sarcomere.

The fact that the RNAi phenotypes for T04A8.4 and F36F2.1 are non-lethal suggested that some form of genetic buffering is likely occurring. To investigate the cause of this phenotypic robustness I chose 13 known muscle-affecting genes whose mutant and RNAi phenotypes were non-lethal with the exception of hlh-1(cc561). Furthermore, I specifically chose genes that had similar annotated functions and expression patterns as the CeSTARS genes. Several of the genes that were queried in this assay were previously demonstrated to affect some aspect of actin dynamics (UNC-87, UNC-22/Twitchin, CDC-42/Rho GTPase, ATN-1/α-actinin, CPN-3, UNC-120/SRF), others are structural proteins that localize to Z line and M line analogs (UNC-95, UNC-97, UNC-98), and yet others have been shown to affect some aspect of myofilament assembly (MUA-6, UNC-45). Genetic mutations were available for all genes under investigation with the exception of F36F2.1. To screen for synthetic genetic interactions, RNAi of the 13 nonessential genes was performed on T04A8.4(gk355) worms and phenotypes were scored to generate a synthetic interaction matrix (Fig’s. 13, 14). I found that T04A8.4 forms a genetic interaction with unc-22 and mua-6 as evidenced by the enhancement of phenotypes observed for both of these genes. Feeding unc-22(RNAi) to T04A8.4(gk355) worms results in a reduction of brood size relative to T04A8.4 worms on control plates. The reciprocal RNAi assay results in an even more pronounced reduction in offspring for unc-22 worms. Similarly, mua-6(RNAi); T04A8.4(gk355) are sterile whereas the reciprocal RNAi of mua-6 worms results in enhanced embryonic
lethality (30%). Two other potential interactions were identified for T04A8.4. Both *unc-89* and *unc-95* show synthetic lethality when fed T04A8.4 dsRNA. In these two cases however, the reciprocal RNAi yields no detectable phenotype.

A synthetic lethal screen was also preformed for F36F2.1. This gene does not currently have an allele available, therefore query strains were fed dsRNA corresponding to F36F2.1, but the reciprocal RNAi was not carried out. Several genes were flagged as possible genetic interactors with F36F2.1 including *unc-22*, *unc-95*, *hlh-1* and *cdc-42*.

*cdc-42* encodes a Rho GTPase that has been previously shown to be expressed at hypodermal cell boundaries (Chen et al. 1996) and is required for activating Rho signaling. In turn, Rho signaling has been shown to be important for monitoring muscle gene expression and myogenesis. In mammals, RhoA has been shown to activate SRF transcription, thereby stimulating the myogenic regulatory factor MyoD (Copeland et al., 2002; Gineitis et al., 2001; Kuwahara et al., 2005). Arai et al. (2002) speculated that STARS may function in striated muscle to stimulate SRF activity through a mechanism that involves RhoA signaling. This hypothesis is supported by work from Hikita et al. (2005), which demonstrated that *cdc-42*(RNAi) caused worms to have locomotory defects resulting from fracturing of consecutive actin filaments in body wall muscle, a phenotype analogous to T04A8.4(gk355). It is interesting then that F36F2.1 may form a synthetic genetic interaction with the Rho GTPase CDC-42. Another potential interaction to examine would be with the muscle Rho guanine exchange factor (GEF), UIG-1 (Hikita et al., 2005).
4.1.1.4 Limitations of the muscle expressome-wide screen:

This screen for novel muscle-affecting genes, though quite sensitive, had several potential drawbacks. Firstly, many of the genes that we screened displayed partial or incomplete penetrance. This is a commonly observed phenomenon with RNA-mediated silencing (Kamath et al., 2003). There are several factors that can affect the sensitivity of a gene to RNAi. For example, some proteins have extended half-lives, and thus targeted depletion of the mRNA does not correlate well with a decrease in the overall abundance of the protein (Montgomery et al., 1998; Ngo et al., 1998; Kamath et al., 2003). In addition, feeding bacterial clones expressing target-gene-specific dsRNA is a less efficient means of knocking-down some gene transcripts (Timmons and Fire, 1998). This differential sensitivity of some genes to RNAi made scoring a clone as ‘muscle-affecting’ or wildtype difficult. For this reason, we assigned each screened gene into a class based on the proportion of animals on a slide displaying a defective myofilament phenotype.

Another difficulty we encountered was the result of age-related sarcopenia, or the gradual loss of muscle mass with time. We noticed an acute loss of myofilament integrity as the worms aged (Fig. 4, 15). Thus, our ability to discriminate between ‘affected’ and ‘non-affected’ worms was compromised if we allowed worms to progress beyond the young adult stage (Fig. 4). Consequently, all worms were screened in L4 and young adult stage.

A further complexity resulted from mosaicism and strain effects of *C. elegans* strain RW1596. These worms are homozygous null for *myo-3*, a gene that is required for persistence beyond the two-fold stage of embryonic development. This strain is rescued by an extrachromosomal array containing a full-length wildtype copy of *myo-3* fused in
frame to GFP. Since the rescuing construct is not integrated into the genome it is lost at a low frequency in some animals. The earlier in development the construct is lost the more severe the phenotype. For example, some embryos fail to receive a copy of the array resulting in a significant proportion of pat embryos. In these instances it is impossible to determine whether a given gene has the effect of disrupting elongation at the two-fold stage or if the pat embryos resulted from a background loss of the extrachromosomal array. If the construct is lost during embryogenesis in a muscle progenitor cell the result will be either loss of muscle cells or disorganized muscle sarcomeres, thereby making scoring of overt phenotypes more difficult (i.e. lineage abnormalities). We attempted to address this problem by integrating the extrachromosomal array into the genome of N2 worms. A line of integrants was obtained, but these worms had significant sarcomeric defects analogous to irregularities observed in mature animals (data not shown). Perhaps overexpression of myo-3 is detrimental to the structural integrity of the myofilaments. It is also conceivable that the construct integrated into, or near, a gene important for sarcomeric maintenance, although the former explanation is more likely than the latter.

The genetic background of C. elegans strain RW1596 represents a dual limitation and potential advantage. In C. elegans, myosin rods assemble tightly around a core of paramyosin molecules. This tight packaging is essential for the apposite functioning of the contractile apparatus. Yet, this strain is homozygous null for myosin heavy chain A (myo-3), made viable by a rescuing extrachromosomal array containing myo-3 fused in frame to GFP, a bulky, tubular protein composed of 238 amino acids (Chalfie et al., 1994). Fusing GFP to the C-terminal end of MYO-3 may affect the compact packaging of myosin rods by preventing tight associations between adjacent units, thereby making
this strain hypersensitive to RNAi of muscle genes. This may have been advantageous, as it may have allowed us to identify genes that only subtly affect sarcomere integrity. On the other hand, using a hypersensitive strain may be a caveat, as one could argue that a reduction in function of two unrelated genes can affect the viability, and in this case the myofilament anatomy, of the RNAi-treated animals (sick plus sick equals sicker).

The muscle 'expressome' was generated by combining available expression data from the *C. elegans* SAGE project and microarray data from the lab of David Miller. This list, though extensive and reliable, is not complete. Several genes previously shown to be expressed in muscle and known to affect sarcomere integrity were not included in the list because they did not appear in either one of two SAGE FACS-sorted muscle replicates or two of three microarray replicates. These include genes that are expressed post-embryonically, such as UNC-22/Twitchin whose expression is only detectable beginning in the L1 stage of development. In addition, most annotated ribosomal and mitochondrial genes were removed from the list. It is entirely possible that some of the genes may function to maintain the integrity of the sarcomere, but we felt that it would be a negligible few, if any. Finally, the muscle expressome screen was not a genome-wide screen for novel muscle-affecting genes; rather this was a comprehensive RNAi screen for new targets for mutational analysis. Thus, we deliberately introduced a limitation on our approach by not screening the entire complement of *C. elegans* genes. We believe that this was a reasonable limitation given that a genome-wide screen would be far more laborious and likely yield only a hand-full of additional muscle-affecting genes over a tissue-specific screen. The result of this decision is that the RNAi screen missed all genes
that affect muscle integrity through extrinsic muscle interactions (i.e. hypodermal-expressed proteins).

Despite the potential limitations of this screen, we are convinced that the precautions that were taken were sufficient to eliminate most, if not all, latent false-positives. By labeling a gene as 'muscle-affecting' only if greater than 50% of animals on a plate had aberrant GFP localization in each of two screens, a phenomenon that was not encountered in negative control worms, we ensure that all putative muscle-affecting genes affect some aspect of myofilament integrity.
CONCLUSIONS

The purpose of this thesis was twofold: to identify novel muscle-affecting genes for future research and to apply the results from the screen to characterize unique genes with close human homologs. To this end, we successfully identified 121 genes that function in building or stabilizing the myofilament lattice, 99 of which had not previously been shown to affect muscle. Many of the genes we identified in this screen were previously shown to affect the structure of the contractile apparatus (15), but we also uncovered a large proportion of novel genes with no known function (48) (Fig. 17). This work, and the online database, should prove to be an excellent tool for researchers interested in studying myofibrillogenesis or, for that matter, FA-like structures in C. elegans.

The other half of the research reported in this thesis pertains to two homologs of mammalian STARS. I have demonstrated that both CeSTARS proteins likely affect myosin stability in sarcomeres, either directly or indirectly. As well, T04A8.4 was shown to be required for proper actin bundle formation in body wall muscle. A putative null allele of T04A8.4 exhibits decreased actin staining and large fractures in the consecutive actin microfilaments in muscle.

Preliminary results from microinjecting full-length GFP::CeSTARS translational reporter fusions suggests that overexpression of these genes results in lethality. More research is required to confirm these results. Although these results are not conclusive, it is still interesting to note the effect of injecting these translational constructs into
wildtype worms. Few F2 roller worms were obtained after microinjecting T04A8.4::GFP (n=2) or F36F2.1::GFP (n=0) into N2 or T04A8.4(gk355) worms (n=4). Of the F2 roller worms obtained, it was quite evident that the worms are not healthy. They showed uncoordinated activity, sluggish movement and vacuolization of the internal body cavity. This is a sharp contrast to another construct, pNAD3 (an unrelated full-length construct), that yielded dozens of F1 roller worms after comicroinjection of the translational reporter construct with a pRF4 (rol-6) dominant marker. Attempts to get F2 roller lines was highly successful (>200 F2 roller worms obtained); however, pNAD3 injected worms did not express the translational GFP reporter. Thus, we can conclude that there is some feature of the CeSTARS::GFP constructs that makes transformed worms ill. Additional experiments are necessary to see if injecting a smaller volume of construct:rol marker has any effect on the viability of the transformed worms. As well, by fusing the CeSTARS full-length constructs to a heat shock promoter we can determine the effect of overexpression of STARS on the viability of transformed worms.

Finally, a screen for synthetic interactors with CeSTARS identified possible genetic modules with unc-22 and T04A8.4, and in the case of F36F2.1, a possible interaction with the muscle Rho GTPase, CDC-42. UNC-22/Twitchin is a large sarcomeric protein with several KOG domains, including an actin-binding domain. The mammalian STARS protein has been shown to bind actin at its C-terminal domain (Arai et al., 2002; Kuwahara et al., 2005), a domain that is both necessary and sufficient for its both actin-binding and for its activation of SRF signaling (Arai et al., 2002). What possible interaction may exist between T04A8.4 and UNC-22? Does UNC-22 act in a
similar or related signaling cascade as CeSTARS to drive the expression of SRF target genes? This assay should provide a framework for future research into this relationship.

SRF/UNC-120 is a MADS-box transcription factor that governs genes involved in cell proliferation, migration, cytoskeletal dynamics, and myogenesis (Miano, 2003; Kuwahara et al., 2005). The CDC-42 protein has been implicated in the activation of SRF transcription in mammalian systems, and Rho signaling in muscle appears to be at least partially dependent on STARS (Arai et al., 2002). Thus, it is reasonable to suggest that a simultaneous reduction of function for STARS and CDC-42 could result in severe defects in muscle actin bundling, thereby leading to embryonic lethality. If the enhancement in lethality observed for the synthetic interaction between CDC-42 and F36F2.1 can be confirmed, it may imply that F36F2.1 is in fact acting analogously to mammalian STARS, mediating F-actin polymerization by depleting G-actin pools through an indirect interaction with a Rho signaling event.

Integrating the results obtained in this thesis with previously published data (Arai et al., 2002; Kuwahara et al., 2005) provides sufficient data to propose a model for CeSTARS function in nematodes: Actin-mediated stimulation of T04A8.4, or perhaps F36F2.1, results in the CDC-42-dependent activation of Rho signaling and the subsequent transcriptional activation of SRF/UNC-120 targets. One target of SRF/UNC-120 is the myogenic inducer *hlh-1* (Fig. 20).
Fig. 20. A model for the action of CeSTARS, T04A8.4 and F36F2.1, in actin dynamics and SRF signaling. Based on data obtained in this thesis and previous results from Arai et al. (2002), we can speculate that T04A8.4 promotes F-actin polymerization by stimulating Rho, which also promotes F-actin polymerization. Rho then activates other effectors, which in turn activate SRF/UNC-120. SRF signaling has been shown to promote myogenesis by activating the bHLH transcription factor, HLH.
REFERENCES


