AN INVESTIGATION INTO THE GENES MEDIATING MYOBLAST MIGRATION IN THE NEMATODE
*Caenorhabditis elegans*

by

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ABSTRACT

During *C. elegans* embryogenesis, myoblasts initially form two rows along the left and right lateral midlines and at ~290 min of development migrate dorsally and ventrally to form the four muscle quadrants present upon hatching (Sulston et al, 1983). As the myoblasts migrate they are still dividing, as are many other cells in their immediate environment. This means the cell-cell contact of cells during migration is dynamic and can vary from animal to animal (Schnabel et al, 1997). This situation creates an environment where the extracellular matrix (ECM) and cell surface contacts are in constant flux, which begs the questions as to how these cells navigate unerringly to their final destination.

In an attempt to identify genes mediating these migrations, I performed an RNAi based screen targeting 776 genes predicted to be members of the extracellular matrix (ECM), or one of its receptors. Using both feeding and injection based RNAi, I was able to identify three genes of interest. Knockdowns of F56B3.2 resulted in paralyzed animals with detached muscle, making it a good candidate for a new component of the muscle attachment complex. F33G12.4 knockdowns resulted in an embryonic arrest phenotype with an abnormal muscle lineage, possibly stemming from polarity defects. The only knockdown that resulted in muscle migration defects was that for *lam-2*, which encodes for the laminin gamma subunit. Analysis of the *lam-2* knockdown, as well as knockdowns for the other laminin subunits, revealed dorsal/ventral migration defects as well as a posterior displacement of the anterior-most ventral muscle
cells. Investigation of this posterior displacement has led to the identification of a previously un-described anterior muscle migration event and its dependency upon the extension of muscle processes from the leading cells.
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LIST OF ABBREVIATIONS

~ Approximately
° Degrees
% Percent
4-D Four-dimensional
BOC Brother of CDO
C Celsius
CAM Cell adhesion molecule
CDO CAM-related/down-regulated by oncogenes
DNA Deoxyribonucleic acid
DEPC Diethylpyrocarbonate
dsRNA Double stranded RNA
ECM extracellular matrix
F2 Second generation
FGF Fibroblast growth factor
GFP Green fluorescent protein
hr Hour
IPTG Isopropyl-beta-D-thiogalactopyranoside
kb kilobase
L Lennox
L1 First larval stage
L4 Fourth larval stage
min Minute
ml Milliliter
mM Millimolar
MRF Myogenic Regulatory Factor
NCAM Neural cell adhesion molecule
ng Nanogram
NGM nematode growth medium
Po Parental generation
PCR Polymerase chain reaction
PDGF Platelet derived growth factor
RNA Ribonucleic Acid
RNAi RNA interference
µg Microgram
µm Micrometer
µl Microliter
x Times
I would like to thank a number of people who helped me get to where I am today. To begin with I would like to thank my parents who supported me through my undergraduate degree, where I apparently learned more than just how to shotgun a beer. Thanks are also due to Jennifer Klenz, without whom I would probably still be working night stock. I would like to thank Don Moerman for giving me my first science job and taking me on as a graduate student. Thanks are also in order to all the members of the lab who have suffered through the few ups and many downs of this research with me. Lastly I would like to thank my darling Allison who has had to put up with a fair share of my irritability over the course of writing this thesis and somehow managed not to kick me to the curb.
1 INTRODUCTION

1.1 Cell migration

Cell migrations play an important role in the proper structuring of embryonic tissues. As fertilized zygotes divide, the nascent cells begin to order themselves, with the first major organization process being gastrulation. During gastrulation, the presumptive endoderm and mesoderm are enveloped by the ectoderm to form a three-layered embryo (Keller 2005). This process is mediated through directional cell migrations in response to external guidance cues. These signals can be either substrate-attached or diffusible. In mesodermal migration during gastrulation both types of signaling play a role. Work done in *Xenopus* and *Drosophila* has found that, early in gastrulation, matrix-bound platelet derived growth factor (PDGF) promotes mesoderm migration along the blastocoel roof, while later on fibroblast growth factor (FGF)-mediated chemotaxis is required to recruit the presumptive somatic cells (Yang et al. 2002; Nagel et al. 2004).

Migratory events continue throughout embryogenesis and become more complex as development progresses. This complexity is exemplified in the nervous system, as the proper wiring of hundreds of millions of neurons is required (Ghashghaei et al. 2007).

Cell migration is performed through dynamic remodeling of the cytoskeleton and occurs in a series of ordered steps. An extracellular signal starts the migratory machinery and can serve as an attractive or repulsive agent. A pro-migratory signal causes localized actin polymerization at the plasma...
membrane in the direction that the signal was detected. As the actin filaments elongate, they force the plasma membrane forward and cell receptors, including integrin and cadherin, anchor the extended membrane to the surrounding ECM and neighboring cells. At the opposite end of the cell, cytoskeleton depolymerization, cell detachment (due at least in part to mechanical shearing), and a myosin-II dependent contraction occur. This contraction is important for cell retraction, but also serves to increase internal hydrostatic pressure. This pressure creates another forward driving force, moving internal components in the direction of migration and at the same time organelles are being trafficked forward along microtubules. The net effect of these steps is forward motion. In the case of a repulsive agent, the extracellular signal results in a depolymerization of actin filaments at the site of signal contact, preventing further migration in that direction (Hynes 2002; Pollard and Borisy 2003; Ridley et al. 2003; Nelson and Nusse 2004; Giannone and Sheetz 2006).

Defects in cell migration have been linked to a number of human diseases, including atherosclerosis and cancer. One of the causes of atherosclerosis is the excess recruitment of vascular smooth muscle cells. These cells secrete ECM components, normally required for proper vascular function. Over-recruitment results in an excess of matrix components being deposited, leading to the hardening of the arteries (Hultgardh-Nilsson and Durbeej 2007). Defective cell migrations also are implicated in cancer. A key stage in cancer progression is metastasis, whereby tumor cells delaminate and migrate away from the primary tumor to other tissues, where they can form new tumors,
severely decreasing patient survivability. A number of genes involved in cell migration have already been linked to metastasis (reviewed by Sahai 2007) and further study of cell migration may lead to the identification of new potential drug treatment targets, or early detection markers.

Understanding cell migration may help in the treatment of non-migratory based diseases as well. Myoblast transfer therapy is a treatment being developed to repair diseased, or damaged skeletal and cardiac muscle (reviewed by Menasche 2007). It consists of isolating and culturing healthy myoblasts and then injecting them into the affected tissue. While it has shown some promise in clinical trials, there are still a number of problems, one of which is that the injected myoblasts fail to migrate from the site of injection (Boldrin and Morgan 2007; Peault et al. 2007). By studying how muscle cells migrate normally in vivo we may identify ways to improve the efficacy of this treatment. Investigating how myoblasts migrate will also shed light on the migratory process in general.

1.2 Caenorhabditis elegans as a model organism

C. elegans is a free living species of nematode, a common soil roundworm found around the globe. It was developed as a genetic tool by Sydney Brenner, who took advantage of its ease of cultivation, short generational time and ability to propagate by mating or self-fertilization to study animal behavior and development. This research ultimately culminating in his becoming a Nobel laureate (Brenner 1973, 1974). Over the years, further research has cemented C. elegans’ status as a major model organism. It was the first multicellular organism to have its genome sequenced and a large research community has lead to the
production of thousands of mutant strains (Consortium 1998). It also has the benefit of an invariant cell lineage, which led to the complete mapping of all cell fates (Sulston and Horvitz 1977; Sulston et al. 1983).

More recently, the development of RNA interference (RNAi), a method by which double stranded RNA (dsRNA) is introduced into an organism leading to a systematic knockdown of its target gene, has been developed in *C. elegans*. The dsRNA can be introduced via microinjection (Fire et al. 1998), by soaking animals in buffer containing dsRNA (Tabara et al. 1998), or by feeding them with bacterial strains expressing the dsRNA of interest (Timmons and Fire 1998), with injection resulting in the highest penetrance (Fortunato and Fraser 2005). An RNAi feeding library with dsRNA expressing bacterial clones targeting ~90% of *C. elegans* genes is available (Kamath et al. 2003), and is an useful tool for performing genetic research.

Approximately 40% of *C. elegans* genes have human homologues (Ahringer 1997; Blaxter 1998). This similarity, combined with all the genetic tools available, makes the worm an attractive system in which to study many biological processes. What makes *C. elegans* an especially attractive organism to study cell migration, is its transparent body and eggs. This allows for the easy visualization of internal tissues and cells, facilitating the observation of cell migrations *in vivo*.

### 1.3 *C. elegans* body wall muscle

*C. elegans* body wall muscle consists of 95, mono-nucleated, striated muscle cells arranged symmetrically in four quadrants, a ventral right, ventral left
and a dorsal left and right quadrant (Sulston and Horvitz 1977). 81 of these muscle cells are derived embryonically, with the remainder arising from a posterior mesoblast cell later in development, the mature muscle cells do not divide (Sulston and Horvitz 1977; Sulston et al. 1983).

As in vertebrates, striated myogenic fate in nematodes is specified by a group of basic helix-loop-helix proteins known as the myogenic regulatory factors (MRFs). In mammalian systems, four factors are required MyoD, Myf5, Myogenin and MRF4 (Ott et al. 1991; Pownall and Emerson 1992; Sassoon 1993; Kassar-Duchossoy L 2004). In *C. elegans* there is only one MRF, HLH-1, the *C. elegans* MyoD homologue. Nematode muscle fate also depends on a pair of other myogenic factors, UNC-120/SRF and HND-1/HAND (Fukushige et al. 2006). Interestingly, these genes play prominent roles in regulating smooth and cardiac muscle development in mammalian systems (Cserjesi et al. 1995; Niu et al. 2005). This has lead to the postulation that all major muscle types, skeletal, smooth and cardiac, may have arisen from a common ancestral cell type (Baugh and Hunter 2006; Fukushige et al. 2006). So while less complex, *C. elegans* muscle is functionally similar to that of higher eukaryotes.

### 1.4 Muscle migration in *C. elegans*

During *C. elegans* embryogenesis 81 muscle cells arise from four of the six so-called “founder cell” lineages, 32 from the C lineage, 28 from the MS lineage, 20 from the D lineage, the only muscle exclusive cell lineage, and 1 from the AB lineage (Sulston et al. 1983). The newly formed myoblasts initially are arranged in two quadrants at the midline on the left and right sides of the embryo,
lying beneath the hypodermal seam cells (Sulston et al. 1983). Starting at ~290 min of embryogenesis, cells in these two quadrants, responding to some cue, migrate dorsally, or ventrally to lie beneath the hypodermal cells and form the final four quadrants present upon hatching (Figure 1) (Sulston et al. 1983; Hresko et al. 1994). The myoblasts’ decision to migrate dorsally or ventrally appears to be lineage dependent (Sulston et al. 1983). Interestingly, these migrations occur while some myoblasts are still dividing and in a dynamic environment where cell-cell contacts differ from embryo to embryo (Schnabel et al. 1997). While the broad stages of C. elegans myoblast migration are well understood, the genes mediating it are still unknown.

Mammalian muscle migration is substantially more complex. The majority of skeletal muscle is derived from progenitors present in the somites, which arise from segmentation of paraxial mesoderm present to the sides of the notochord. Environmental signals cause the specification of myogenic and dermal progenitors in the dorsal somite forming a structure known as the dermomyotome. As myogenic fate is determined, the myogenic cells delaminate and migrate from the dermomyotome to the myotome, where they fuse to form myotubes. Delaminated myogenic cell can also migrate from the dermomyotome directly to sites of limb bud formation (Kalcheim C 1999; Buckingham 2001; Christ and Brand-Saberi 2002). One of the first proteins identified as affecting muscle migrations was the extracellular protein laminin, which promotes myoblast migration in vitro (Foster et al. 1987; Goodman et al. 1989). Since then, a number of pro-myogenic factors have since been identified.
Figure 1: *C elegans* embryonic muscle migrations. Panel A depicts a ~5hr embryo with muscle cells adjacent to the hypodermal seam cells (modified from Hresko et al 1994). Arrows indicate the direction of migration. B-E are anti-CeMyoD antibody staining of embryos undergoing muscle cell migration (D. Moerman, unpublished). Panels are of increasingly older embryos and show the dorsal and ventral migrations that generate the final four muscle quadrants.
in mammals, including cadherins, CDO (CAM-related/down-regulated by oncogenes), BOC (brother of CDO) and neogenin. Though mutations in these genes appear to cause defects in promoting muscle cell fate, the majority of cells still migrate properly (reviewed in Krauss et al. 2005). Most recently, in vivo experiments in zebrafish have identified that differential expression of N-cadherin and M-cadherin are required for the proper orchestration of different waves of myoblast migration to the myotome, while in quail, N-cadherin mediated adhesion is required for proper myotome colonization (Cortes et al. 2003; Cinnamon et al. 2006). While progress is being made in the field, many factors mediating muscle migratory events still remain elusive. By studying this process in a simpler organism, such as C. elegans, we hope to identify new conserved genes regulating this process.

1.5 Known C. elegans muscle attachment proteins

A good place to start looking for proteins that might be mediating muscle migration in C. elegans is amongst those that are involved in muscle attachment. C. elegans muscle cells are attached to the surrounding ECM via $\alpha/\beta$ integrin based attachment complexes (Figure 2). Starting at the muscle cell surface, the PAT-2/PAT-3/$\alpha/\beta$ integrin heterodimer binds the ECM protein UNC-52/Perlecan (Rogalski et al. 1993; Hresko et al. 1994; Gettner et al. 1995). On the hypodermal side, a transmembrane protein, LET-805/Myotactin, is involved and may play a role in linking the ECM to the fibrous organelle, a structure similar to
Figure 2: Muscle attachment in *C. elegans*. Panel A is a cartoon depicting the four muscle quadrants arranged next to the hypodermis. Panel B is a blow up of the muscle-hypodermis adhesion complex depicting the identified proteins required for proper muscle-hypodermal adhesion and their location. Figure adapted from (Moerman and Williams 2006).
hemi-desmosomes, that carries the linkage across the hypodermis to the cuticle (Hresko et al. 1999). At the cuticle-epidermal interface two transmembrane proteins, MUP-4 and MUA-3, form a link between the hypodermis and cuticle (Bercher et al. 2001; Hong et al. 2001). While mutations in these genes cause severe defects in muscle attachment, usually resulting in embryonic arrest, muscle migrations appear unaffected (Francis and Waterston 1991; Rogalski et al. 1993; Hresko et al. 1999; Bercher et al. 2001; Hong et al. 2001). These previous studies, while not exhaustive, did not identify these genes as mediating myoblast migration, so the focus now must shift to the identification of new genes.

1.6 The ECM as a source for candidate genes involved in muscle migration

Cell migration is a behavior common to most cell types at some point during their development and the fundamental machinery being used should be common among them as well. One class of proteins required for migration that need not be conserved between different cell types is the ECM proteins and their receptors. As mentioned previously, the extracellular environment serves not only as a scaffold for migrating cells, but is also the source of migratory cues (Ridley et al. 2003). As many different cell types share the same environment, they must be able to recognize and selectively respond to the appropriate cues. This necessity makes the ECM and its receptors an attractive protein set to search for tissue specific migration affecting proteins.
1.7 An RNAi screen to identify genes involved in myoblast migration

Though a number of ECM and cell surface molecules have been shown the be involved in cell migration, positioning and attachment, including fibronectin, laminin, type IV collagens, the NCAM, cadherin and integrin families (Levi G 1990; Adams and Watt 1993), and the UNC-6/netrins (Ishii et al. 1992; Serafini et al. 1994), previous analyses have failed to identify any that affect early muscle migration in *C. elegans* (Hedgecock et al. 1990; Guo et al. 1991; Ishii et al. 1992; Leung-Hagsteijn et al. 1992; Rogalski et al. 1993; Sibley et al. 1993; Williams and Waterston 1994; Gettner et al. 1995). In an effort to identify genes affecting *C. elegans* muscle migration, I set about performing a genetic screen using RNAi.

In an attempt to make the screen more efficient, I decided not to do a genome-wide RNAi screen, but chose to only screen a subset, consisting of genes that had been predicted to be a member of, or to interact with the ECM (H. Hutter personal communication). This gave a list of 948 genes, 776 of which were available commercially as RNAi feeding clones and served as the basis of my screen.

Two phenotypes of interest were focused on: miss-localized muscle cells in hatched animals and embryonic lethality. It had previously been shown using laser ablation, that when there were cells missing in a muscle quadrant, the remaining cells could elongate to compensate (Moerman et al. 1996). In those instances, even if the animal was missing up to a quarter of its muscle cells, it
could still appear, for all intents and purposes, wildtype. That being the case, a strain harbouring a muscle specific green fluorescent protein (GFP) (PD4251) was used to visualize the muscle cells in post-embryonic animals. I hoped that by using this fluorescent strain, I would be able to detect muscle defects that had been missed in previous screens that focused on morphology. Embryonic lethality was also screened for. Severely defective muscle migrations should result in embryonic arrest, as previously identified mutations in a number genes involved in muscle attachment to the ECM result in embryonic arrest (Rogalski et al. 1993; Williams and Waterston 1994; Gettner et al. 1995). To visualize the muscle in the arrested embryos, a strain with GFP labeled myoblasts was used (PD7963). In all, six gene knockdowns resulted in post-embryonic muscle positioning defects and nine induced embryonic lethality.

To better understand what was occurring in the arresting embryos, a subset were further analyzed using 4-D microscopy (Schnabel et al. 1997; Thomas and White 1998). This detailed analysis resulted in the identification lam-2, which encodes the laminin gamma subunit, as being required for proper muscle migrations. These migratory defects hold true for RNAi knockdowns of any of the laminin subunits. Another benefit of this type of analysis is the ability to identify cell lineage defects, which lead to the identification of F33G12.4 as being required for proper muscle cell fate. Knockdown of this gene resulted in a number of muscle fate changes, with the loss of muscle cells in some lineages and gain of muscle cells in others. These widespread defects point to F33G12.4 as being important in cell fate determination early on in embryogenesis.
Of all the results to come out of the 4-D analysis, the most interesting was the observation of an anterior muscle migration event that occurred after the dorsal and ventral muscle cell migrations had finished. This anterior migration had not been previously reported in prior descriptions of muscle cell migration (Sulston et al. 1983; Hresko et al. 1994; Schnabel et al. 1997) and presents an interesting phenomenon for further study.
2 Materials and Methods

2.1 Strains Used

Two strains were used for all the experiments. PD4251, ccls4251 I; dpy-20(e1282) IV, with the integrated array (ccls4251) containing three constructs: pSAK2 (a myo-3 promoter driving a nuclear-targeted GFP-LacZ fusion), pSAK4 (myo-3 promoter driving mitochondrially targeted GFP), and a dpy-20 subclone. The array rescues dpy-20(e1282). In this strain, under fluorescent light, muscle cells are clearly visible, with both their nuclei and mitochondria fluorescing green.

As the myo-3 promoter does not turn on until late in embryogenesis, another strain, PD7963, was used to visualize the myoblasts earlier in embryogenesis. PD7963 contains an integrated construct containing an hlh-1 promoter driving a nuclear-targeted GFP and labels nascent myoblasts for the duration of embryogenesis. Both PD4251 and PD7963 were gifts from Dr. Andrew Fire.

2.2 Gene set selection

A list of genes predicted to be either in the ECM, or a cell surface receptor in the worm had previously been generated (Harald Hutter, personal communication). The list was compiled by searching for genes containing at least one of 46 known ECM and receptor protein domains (Table 1) in their predicted
Table 1: List of all protein domains known to be associated with ECM proteins and receptors. Note that genes may contain more than one protein domain.

<table>
<thead>
<tr>
<th>Protein Domain</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM/Reprolysin zinc metalloprotease</td>
<td>11</td>
</tr>
<tr>
<td>Amiloride-sensitive Na+ channel</td>
<td>31</td>
</tr>
<tr>
<td>Anaphylatoxin</td>
<td>1</td>
</tr>
<tr>
<td>Cadherin</td>
<td>15</td>
</tr>
<tr>
<td>SEA</td>
<td>5</td>
</tr>
<tr>
<td>C-terminal Cystine Knot</td>
<td>8</td>
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<tr>
<td>C-type Lectin</td>
<td>265</td>
</tr>
<tr>
<td>Sushi/CCP/SCR</td>
<td>13</td>
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<tr>
<td>CUB domain</td>
<td>93</td>
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<tr>
<td>Cystatin</td>
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<tr>
<td>Disintegrin</td>
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<tr>
<td>Delta Serate</td>
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<td>Epidermal Growth Factor like</td>
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<tr>
<td>Coagulation Factor 5/8C</td>
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<td>Fibrinogen beta/gamma C-terminal</td>
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<tr>
<td>Galectin, (S-type) lectin</td>
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<td>Immunoglobulin</td>
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<td>Kringle</td>
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<td>Kunitz/BPTI</td>
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<td>Laminin IV (B-Type)</td>
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<td>LDL receptor A</td>
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<td>Laminin IV (A-type)</td>
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<td>Matrixin</td>
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<tr>
<td>Nidogen N-terminus</td>
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<td>Notch domain</td>
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<td>P-type/Trefoil</td>
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<td>SEA</td>
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</tbody>
</table>
protein sequences. This provided a list of 948 genes enriched for, but not exclusively containing, ECM proteins and their receptors. 776 of these genes already had RNAi feeding constructs available and were the basis of my screen.

2.3 RNAi feeding library

RNAi knockdown was performed using an RNAi feeding library created in Dr. July Ahringer’s lab at the University of Cambridge (Cambridge, UK). It is available from Geneservice (UK) and contains ~17,000 frozen bacterial clones, covering 87% of the predicted C. elegans genes (Kamath et al. 2003). Each clone consists of a genomic fragment of a gene inserted into a specialized feeding vector. 1-2 kb genomic fragments were polymerase chain reaction (PCR) amplified using primers generated by Research Genetics Genepairs. Amplicons were then cloned into a EcoRV digested L4440 (pPD129.36) vector, which inserted the cloned fragment between bi-directional T7 promoters (Timmons and Fire 1998). Finally, purified constructs were transformed into HT115(DE3) bacterial cells, which lack dsRNA specific RNase III activity, and frozen.

2.4 RNAi feeding experimental procedure

2.4.1 Preparing worms for feeding

Worms were grown on nematode growth medium (NGM) petri dishes seeded with Escherichia coli (OP50 strain), to a high density. Worms were
washed from the plate using M9 buffer into 50 ml polypropylene tubes. Worms were then pelleted by centrifugation and the supernatant was removed. 40ml of hypochlorite solution (75% dH₂O, 20% sodium hypochlorite, 5% 10 N KOH) was added to the worm pellet and shaken until no worm carcasses were visible in the suspension. Embryos were pelleted and rinsed 3 times in 40ml of M9 buffer. After the final rinse, embryos were re-suspended in 10ml of M9 into a clean 15ml polypropylene tube and incubated overnight at room temperature with gentle shaking. This allowed for the isolated embryos to hatch and synchronized the population at the first larval stage (L1).

2.4.2 RNAi feeding

Individual clones were taken from the Geneservice feeding library and cultured overnight in Lennox (L) broth containing 50 µg/ml ampicillin. Modified NGM plates, containing 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to induce dsRNA synthesis in the clones, and 50 µg/ml cabenicillin to select for bacterial containing the RNAi construct, were prepared. These plates were streaked with 50 µl of overnight culture and incubated overnight at room temperature to allow for bacterial growth and dsRNA production.

Approximately 5-10 of the previously prepared L1s were aliquoted onto each RNAi feeding plate. The plates were then incubated at 20 ºC until they began to lay eggs (~60 hrs) at this time the adults were removed. The remaining eggs and larvae were incubated at 20 ºC until they began to lay eggs (~60 hrs) and two of the gravid young adults were transferred to a fresh plate, allowed to
lay eggs overnight and removed. The freshly laid eggs were allowed to develop until they reached the fourth larval stage (L4) (~36 hrs) and then screened. All feedings were done in duplicate and in some cases worms died/failed to propagate and were screened at earlier time points.

2.4.3 Morphological screen

After reaching the L4/young adult stage, F2 worms were screened for visible phenotypes using a dissecting microscope (Wild Heerbrugg model). These included embryonic lethality, larval arrest, uncoordinated movement, paralysis, slow growth, ruptured vulva, sterility and body morphology defects. Feeding constructs causing embryonic lethality were noted for further embryonic lethal analysis.

2.4.4 Visualization of muscle cells

F2 worms were prepared for analysis by transferring 20-50 animals into a 12 ml drop of M9 solution containing 10mM sodium azide on a glass slide. Cover slips were then gently applied to prevent the rupturing of the paralyzed worms. The slides were then analyzed under fluorescent light on a Ziess Axioplan compound microscope. Worms were viewed under 200x and 400x magnifications and images on any displaced or missing muscle were taken using a Qimaging QICAM digital camera running Qcapture version 1.68.4. Slides showing defects in a least 10% of the animals were counted as positive and retested.
2.4.5 PCR confirmation of feeding clones

The presence of the correct DNA fragment in the RNAi feeding clones was detected using PCR. Primers specific to the DNA fragments in clones that caused a phenotype were used in 25 ml PCR reaction containing 10 pmol of both the forward and reverse primers, 1x PCR buffer, 480 mM dNTPs, 1 unit of Taq polymerase, and DNA from the clone being tested. Products were separated using gel electrophoresis in a 1% TAE agarose gel. PCR fragments were then observed by staining with CyberSafe and visualized using a blue light transilluminator. Clones were deemed correct if the insert specific PCR produced the correct sized band.

2.5 RNAi injection experimental procedure

2.5.1 Gene selection

Injection based RNAi was done on a subset of genes as a means of facilitating 4-D recordings, as injection based RNAi has a much higher penetrance. Most genes selected for RNAi knockdown by injection were taken from the list of those that displayed embryonic lethality when knocked down using RNAi using the feeding clones. A few others were selected because, while they showed a post embryonic phenotype, they had previously been shown to be embryonic lethal in an injection based RNAi screen (Sonnichsen et al. 2005).
2.5.2 dsRNA fragment generation

To generate the dsRNA oligos, primers, with 5' attached T7 promoters, specific to 1-2 kb of the gene of interest were generated. These primers were used in three 50 µl PCR reaction containing 10 pmol of both the forward and reverse primers, 1x PCR buffer, 480 µM dNTPs, 1 unit of Platinum Taq DNA Polymerase Hi-Fidelity (Invitrogen), and genomic DNA. PCR products were then purified by gel electrophoresis in a 1% TAE, low melting point agarose gel. PCR fragments were observed using Sybr Safe DNA Gel Stain (Invitrogen), visualized under a blue light transilluminator and the fragment of interest was cut out. The isolated gel fragment was melted at 72°C and digested using β-agarase (Invitrogen) (1 unit/100 µg gel) at 42°C. The DNA amplicons were then isolated by sodium acetate precipitation.

The purified amplicons were used as templates for the RNA synthesis, using the MEGAscript T7 High Yield Transciption Kit (Ambion). Synthesized RNA was spin column purified using a RNeasy Mini Kit (Qiagen), quantified and a sample run in a 1% TAE agarose gel to confirm correct product size. Purified RNA was aliquoted and stored.

2.5.3 Microinjection procedure

Synthesized dsRNA was introduced into worms via microinjection. The injection mixes contained the dsRNA of interest diluted to 200ng/µl in DEPC treated water and was injected into the gonads of PD7963 worms. Injections were performed on a microinjection setup featuring a Zeiss inverted compound
microscope (IM35) using conventional methods (Mello et al. 1991). Injected adults were then cultured for 24hrs before being used for further analysis.

### 2.5.4 Embryonic lethal analysis

Eggs were manually dissected from gravid adult worms. Isolated embryos were then mounted on 4% agar pads using previously described methods (Sulston et al. 1983). Embryos were incubated at room temperature for at least 10hrs. Using a dissecting microscope (Wild Heerbrugg model), the slides were screened for arrested embryos. Slides exhibiting at least 10% arrested embryos were then subjected to fluorescent microscopy as described previously.

### 2.5.5 4-D microscopy and lineage analysis

2-4 celled embryos were isolated from RNAi treated gravid adults. Two isolated embryos were manipulated so that they were next to each other in the same orientation and mounted using conventional methods (Sulston et al. 1983). These slides were then used for 4-D (multi-focal, time-lapse) recordings, on a Zeiss Axioplan microscope. Embryos were recorded at 25 °C with Z-stacks, using Nomarski microscopy, taken every 30–45 s over a 7-hr time course. Interspersed with the Nomarski Z-stacks, several fluorescent Z-stacks were taken to visualize the GFP expressing muscle cells (Moerman et al. 1996; Schnabel et al. 1997; Thomas and White 1998; Burglin 2000). For data analysis
we used Simi Biocell, a program designed to analyze *C. elegans* 4-D embryo recordings (Schnabel et al. 1997).
3 Results

3.1 RNAi screen of predicted ECM proteins and receptors

To search for genes involved in muscle migration I performed a reverse genetic screen using RNAi knockdowns. Instead of performing a genome-wide screen, I chose to focus on those genes predicted to encode ECM proteins and receptors. This served two purposes: it made the screen more manageable and filtered out the structural proteins required for cell migration. The initial screen consisted of 776 genes predicted to be members of the ECM, or one of its receptors (Harald Hutter, personal communication). Throughout the screen, a feeding bacterial strain (HT115(DE3)) with a L4440 vector without any insert served as a negative control. Two strains were used as positive controls, a feeding vector against *epi-1*, which encodes a laminin subunit, and one against *ina-1*, which encodes an alpha integrin. Effective knockdown of *epi-1* leads to sterility in the Po generation, indicating the strength of the knockdowns in the first generation. Knocking down *ina-1* shows a less penetrant phenotype, with a proportion of animals rupturing at their vulvae. If fewer than 50% of the *epi-1* knockdown Pos were sterile, or fewer than 20% of the *ina-1* F2s ruptured, the screen was redone.

In the first round of screening the muscle marker strain PD4251 was used and resulted in 15 gene knockdowns exhibiting missing or misplaced muscle cells and 50 that produced a detectable level of embryonic lethality. All of these initial hits were then re-tested.
3.2 Re-screen of genes causing defects in post-embryonic muscle

RNAi feeding clones that resulted in F2 worms exhibiting post-embryonic muscle position defects were re-screened twice, following the same protocol as before. Of the 15 genes that initially showed post-embryonic muscle position defects when knocked down, only 6 displayed a phenotype in 2/3 rounds of testing (Table 2). Included in these are let-805, which encodes a known attachment gene (Hresko et al. 1999), lev-10, which encodes a protein required for proper acetylcholine receptor clustering and hmr-1, which encodes a cadherin protein implicated in hypodermal morphogenesis (Costa et al. 1998). The remaining identified previously uncharacterized genes displayed phenotypes similar to those of either hmr-1 or let-805 (Figure 3-4).

3.3 Re-screening of genes causing embryonic arrest

Knockdowns that caused embryonic lethality were re-tested, using the myoblast marker strain PD7963. Of the 50 knockdowns that initially displayed embryonic lethality 19 retested at a level of at least 10%, with most testing in the 10-11% range. Included in this set are seven genes previously identified as causing embryonic lethality and one false positive, ced-1, which is not required for embryonic viability (Zhou et al. 2001). If the cut off is set at 20%, we lose the known false positive and one of the previously identified lethals and are left with 9 genes (Table 3). Of the remaining known positives, two, zyg-11 and hmr-1,
Table 2: RNAi targeted genes resulting in post-embryonic muscle phenotypes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>E03A3.5</td>
<td></td>
<td>C-type lectin</td>
</tr>
<tr>
<td>F56B3.2</td>
<td></td>
<td>EGF-like domain containing protein of unknown function</td>
</tr>
<tr>
<td>H19M22.2</td>
<td>let-805</td>
<td>Myotactin</td>
</tr>
<tr>
<td>W02B9.1</td>
<td>hmr-1</td>
<td>Classical Cadherin</td>
</tr>
<tr>
<td>Y37E11AL.6</td>
<td></td>
<td>Predicted secreted protein with two cadherin domains and a C-terminal EGF-like domain</td>
</tr>
<tr>
<td>Y105E8A.7</td>
<td>lev-10</td>
<td>Transmembrane protein required for acetylcholine receptor clustering</td>
</tr>
</tbody>
</table>
Figure 3: Post-embryonic muscle coiling RNAi phenotype. A: Wildtype adult. B: hmr-1 C: E03A3.5 D: lev-10. E: Y37E11AL.6. In the knockdowns, the muscle cells appear to wrap around the animals. All panels are fluorescent images depicting GFP labeled muscle cells. Arrows indicate regions of muscle cell coiling. Scale bar in panel A is 100 μm.
Figure 4: RNAi knockdowns of let-805 and F56B3.2. A: Wildtype. B: F56B3.2 RNAi knockdown. C: let-805 RNAi knockdown. The arrows indicate gaps in the muscle quadrants. In all panels the strain is PD4251 and anterior is to the right. Size bars are 100 µm.
were first identified through mutational screens and the genes, K07A12.2, F28B3.1, T09A5.9 and F33G12.4, were identified in previous large scale RNAi screens. K07A12.2 is required for proper osmotic integrity, T09A5.9 is involved in regulating cell cycle, F28B3.1 embryos fall apart during elongation and F33G12.4 has an undefined phenotype, arresting mid-embryogenesis (Carter et al. 1990; Costa et al. 1998; Kamath et al. 2003; Simmer et al. 2003; Sonnichsen et al. 2005).

The remaining knockdowns displayed a range of embryonic arrest phenotypes (Figure 5). In knockdowns of Y116A8C.21 and K04E7.3, embryos appear to fall apart as the embryo elongates. In the case of C44H4.1, embryos appear to deteriorate before elongation has begun in earnest. When looking at the myoblasts in these embryos, it is easy to see that some of them are displaced (data not shown). What is more difficult is determining whether these muscle defects are the cause of the disorganization, or simply a result of other morphogenic defects. In an attempt to gain a better understanding of what is occurring in these embryos, a few were selected to undergo a more detailed analysis, using 4-D microscopy.

### 3.3.1 Detailed embryonic analysis

To get a better understanding of the muscle cell migrations occurring in the arresting embryos, 4-D analysis was performed on a select few of them. To increase the effectiveness of the RNAi, as only two embryos can be recorded at
Table 3: Gene knockdowns resulting in 20% embryonic lethality. For determining percentage embryonic lethal N = 50-100.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Percent Lethal</th>
<th>Concise description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C08B11.1</td>
<td>zyg-11</td>
<td>90%</td>
<td>Leucine-rich repeat containing protein</td>
</tr>
<tr>
<td>K07A12.2</td>
<td></td>
<td>80%</td>
<td>Leucine-rich repeat containing protein</td>
</tr>
<tr>
<td>F33G12.4</td>
<td></td>
<td>59%</td>
<td>Leucine-rich repeat containing protein</td>
</tr>
<tr>
<td>T09A5.9</td>
<td></td>
<td>35%</td>
<td>Protein phosphatase subunit</td>
</tr>
<tr>
<td>W02B9.1</td>
<td>hmr-1</td>
<td>26%</td>
<td>Classical cadherin</td>
</tr>
<tr>
<td>C44H4.1</td>
<td></td>
<td>24%</td>
<td>Leucine-rich repeat containing protein</td>
</tr>
<tr>
<td>K04E7.3</td>
<td>nas-33</td>
<td>22%</td>
<td>Astacin protease</td>
</tr>
<tr>
<td>F28B3.1</td>
<td></td>
<td>20%</td>
<td>Cystatin proteinase inhibitor-like</td>
</tr>
<tr>
<td>Y116A8C.21</td>
<td></td>
<td>20%</td>
<td>C-type lectin</td>
</tr>
</tbody>
</table>
a time, synthesized dsRNA oligos were used and introduced into the animals by gonadal microinjection. Some genes were selected from the pool of genes identified as embryonic lethal in the previous feeding screen (Table 4). A few others were selected by comparing the list of genes that showed a post-embryonic phenotype in the previous screen to a list of genes identified in a genome wide injection-based RNAi screen for genes required for proper embryogenesis (Sonnichsen et al. 2005). The logic behind this was that because RNAi knockdown by feeding is less penetrant than knockdown by injection, genes required for embryogenesis may not have been knocked down sufficiently to result in embryonic arrest. Also, in some cases the RNAi introduced by feeding affected gonadogenesis in the Pos resulting in no eggs being laid at all, preventing analysis of embryogenesis.

Genes selected by the two methods were subjected to injection-based RNAi, recorded using a 4-D microscopy system and those showing muscle phenotypes of interest were analyzed.

### 3.3.1.1 F33G12.4, a lineage-affecting gene

An added benefit of labeling the myoblasts using a muscle specific GFP construct is that we can also detect defects in the cell lineage that affect muscle cells. This was the case for one of the genes, F33G12.4

Initial analysis showed that embryos had delayed cell divisions and arrested early on in embryogenesis. When examined under fluorescent microscopy it was obvious that there were also defects in the position of the muscle cells. Normally by this point in development, muscle cells are arranged in
### Table 4: Genes selected for injection based RNAi

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Feeding Phenotype</th>
<th>Injection Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16A3.6*</td>
<td></td>
<td>Embryonic lethal</td>
<td>Embryonic lethal, early arrest at ~20-30 cell stage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11%)</td>
<td></td>
</tr>
<tr>
<td>C44H4.1</td>
<td></td>
<td>Embryonic lethal</td>
<td>Reduced brood size, some eggs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(24%)</td>
<td>are small and arrest at ~1.5 fold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>stage with no muscle migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>defects</td>
</tr>
<tr>
<td>C54D1.5*</td>
<td>lam-2</td>
<td>Po sterile, 2/2</td>
<td>Embryonic Lethal, muscle migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>screens</td>
<td>defects</td>
</tr>
<tr>
<td>F33G12.4*</td>
<td></td>
<td>Embryonic lethal</td>
<td>Embryonic Lethal, muscle lineage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(59%)</td>
<td>defects</td>
</tr>
<tr>
<td>T13C2.6*</td>
<td></td>
<td>Po sterile, 1/2</td>
<td>Wildtype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>screens</td>
<td></td>
</tr>
</tbody>
</table>

* RNAi resulted in embryonic lethality is a previous injection based screen (Sonnichsen et al. 2005).
two quadrants along the left and right sides of the embryo (Sulston et al. 1983), but in the knockdown embryos the muscle cells had accumulated in a clump in the posterior (Figure 6). When a detailed analysis was performed, it became clear that the defects were not migratory, but originated in the cell lineage.

In the knockdown embryos a number of muscle lineage defects were identified, specifically in the C, P4 and MS lineages. In wildtype embryos the C lineage gives rise to both hypodermal and muscle cells, with the C grand-daughters Caa and Cpa giving rise to hypodermal cells and Cap and Cpp giving rise to muscle (Sulston et al. 1983). In the F33G12.4 knockdown the C lineage solely gives rise to muscle. Normally, the P3 cell gives rise to the muscle exclusive D cell lineage and the gamete generating P4 cell lineage (Sulston et al. 1983), but in the knockdown embryos P4 adopts a fate similar to D and produces muscle cells. Finally, the MS lineage, which contributes 28 muscles, fails to produces any muscle cells in the knockdown (Figure 7). All these defects point to F33G12.4 playing an important role in establishing cell identity early in embryogenesis.

3.3.1.2 lam-2, a muscle migration-affecting gene

In my RNAi feeding screen, a lam-2 knockdown resulted in sterile Po adults, but had been shown to cause embryonic lethality when knocked down using injection RNAi (Sonnichsen et al. 2005). This being the case, lam-2 was one of the genes selected for injection based RNAi knockdown.
Figure 6: Knockdown of F33G12.4 results in mis-localization and a reduced number of myoblasts. A-B wildtype. C-D F33G12.4 RNAi knockdown. A and C are Nomarski images. B and D are fluorescent images displaying GFP labeled myoblasts. In all panels anterior is to the left. Scale bars are 10 μm.
Figure 7: F33G12.4 knockdowns lack MS derived myoblasts. A: Early wildtype MS muscle lineage. B: F33G12.4 lineage of MSappp and MSpppp. In both panels forks represent cell divisions, dots indicate presumptive myoblast cell fates and time is on the y-axis. In B the gray lineage tree represents the expected division pattern and illustrates the delayed cell divisions observed in the F33G12.4 knockdowns. In panel C, 1 and 2 are images of MSappp and 3 and 4 are images of MSpppp at the lineage end point depicted in panel B, illustrating their lack of myogenic fate, as indicated by the lack of GFP expression. 1 and 3 are Nomarski images and 2 and 4 are fluorescent images of GFP labeled myoblasts. In panel B anterior is to the left and the white rectangles indicate the nuclei of MSappp and MSpppp. The scale bar is 10 µm.
When knocked down using the injection protocol, lam-2 resulted in embryonic lethality, with embryos arresting anywhere between the onset of elongation to the two-fold stage of embryogenesis. Analysis of these knockdowns revealed a number of muscle defects. These ranged from improper attachment, to lagging cells along the embryo, and in severe cases a complete absence of the anterior-most ventral muscle cells (Figure 8). Further lineage analysis was performed to determine whether these ventral anterior-most muscle cells were absent in the embryos, or had improperly migrated. Following the migrations of these cells revealed that they were indeed present, but were further to the posterior of the embryo (Figure 9).

While analyzing the lam-2 RNAi knockdowns there was one phenotype of particular interest, the posterior displacement of the anterior-most ventral muscle cells in most of the arrested embryos. This was unusual, as previous studies have only described dorsal and ventral muscle migrations from beneath the hypodermal seam cells to the dorsal and ventral hypodermal cells (Sulston et al. 1983; Hresko et al. 1994; Schnabel et al. 1997). Analysis of pre-muscle migration embryos failed to detect any defects in the anterior-posterior distribution of myoblasts, suggesting the posterior displacement resulted during migration (Figure 10). To better understand this phenomenon, I performed a detailed analysis of the anterior-most ventral muscle cells in wildtype embryos. What I
Figure 8: Knockdown of the lam-2 gene via RNAi results in muscle migration defects. A-B Wildtype 1.5 fold embryo. C-D comma stage lam-2 knockdown embryo. E-F ~2-fold lam-2 knockdown. A, C and E are Nomarski images and B, D and F are the corresponding fluorescent images showing GFP labeled muscle cells. Arrows indicate lagging cells in between the dorsal and ventral muscle quadrants. Triangles indicate missing anterior ventral muscle cells. In all panels anterior is to the right and dorsal is up. Scale bar is 10 µm.
Figure 9: Anterior-most ventral muscle cells are displaced to the posterior in lam-2 knockdowns. A, C and E: wildtype. B, D and F: the lam-2 knockdown. A and B are Nomarski images and C and D are fluorescent images displaying GFP labeled myoblasts. E and F are stick and ball models depicting the migration of the anterior-most ventral cell. In all panels anterior is to the left and dorsal is up. Arrows indicate the anterior-most ventral muscle cell. Scale bar is 10 µm.
Figure 10: Pre-migration muscle cell position appears wildtype in lam-2 knockdowns. A-B: Wildtype. C-D: lam-2 knockdown. A and C are Nomarski images of late gastrula embryos. B and D are fluorescent images showing GFP labeled myoblasts. In all panels anterior is to the left and left is up. Scale bar is 10 µm.
found was that there were anterior muscle migrations occurring and they were happening after the initial dorsal and ventral migrations (Figures 9 and 11). As muscle cells migrate dorsally or ventrally, the anterior most muscle cell of each of the forming quadrants extends a process to the anterior and this process is no longer present after the anterior migrations (Figure 12). In the lam-2 knockdowns we see a loss of this extended process in the ventral quadrants, but not the dorsal ones (Figure 13).

To determine whether this phenotype was a result of disrupting the laminin trimer, or specifically related to the gamma subunit, and to lend support for the phenotype not being the result of the RNAi affecting an off target, RNAi knockdown was performed on lam-1 and epi-1. These genes encode the beta subunit and one of the alpha subunits of laminin, respectively. Knockdown of either gene also resulted in embryonic lethality and the arrested embryos displayed defects in muscle migration similar to those observed in the lam-2 knockdowns (Figure 14).
Figure 11: Anterior muscle migration of anterior-most ventral muscle cell. After completing dorsal or ventral migrations the anterior most muscle cells migrate to the anterior. A-D are Nomarski images of a wildtype embryo. Each image was taken 35s after the preceding one. Arrows indicate the nuclei of the anterior-most, right, ventral muscle cell. In all panels anterior is to the right and dorsal is up. Scale bar is 10 µm.
Figure 12: Muscle arm extension by the anterior-most muscle cells during cell migration. A and C are Nomarski images of the same wildtype embryo, C was taken 30 minutes after A. B and D are fluorescent images showing GFP labeled muscle cells. In all panels anterior is to the right and dorsal is up. Triangles indicate the nuclei of the anterior-most muscle cells. Arrows indicate muscle arms. Scale bar is 10 µm.
Figure 13: Anterior-most ventral muscle cells fail to extend muscle processes in laminin knockdowns. A-B: wildtype. C-D: lam-2 knockdown. A and C are Nomarski images. B and D are fluorescent images of GFP labeled myoblasts. Arrows indicate extended muscle processes. In all panels anterior is to the right and dorsal is up. Scale bar is 10 μm.
Figure 14: Knockdown of the beta subunit, *lam-1*, or the alpha subunit, *epi-1* results in a phenotype similar to *lam-2*. A-B: wildtype. C-D: *epi-1* knockdown. E-F: *lam-1* knockdown. A, C, and E are Nomarski images of ~1.5 fold embryos. B, C, and F are fluorescent images showing GFP labeled muscle cells. Arrows indicate lagging cells and triangle indicate misplaced anterior-most ventral cells. In all panels anterior is to the right and dorsal is up. Scale bar is 10 mm.
4 Discussion

4.1 Genes identified in the screen

In an attempt to identify genes involved in myoblast migration I screened, using RNAi, 776 genes predicted to be a part of, or to interact with, the ECM. The phenotypes I was most interested in were those that displayed post-embryonic muscle defects or resulted in embryonic lethality. In all, I found six genes that reproducibly displayed post-embryonic muscle defects and nine genes that resulted in the arrest of embryos when knocked down.

When examining the post-embryonic muscle phenotypes detected, they appear to fall into two groups: those that are similar to $hmr$-1 and those that have a phenotype like $let$-805. The $hmr$-1 group, consisting of $hmr$-1, E03A3.5, Y37E11Al.6 and $lev$-10, displays a phenotype where the muscle quadrants appear to coil around the body off the animal (see figure 3). This phenotype is similar to that observed in the roller ($rol$) class of genes. These genes are primarily cuticular collagens that when mutated can result in an improperly organized cuticle, resulting in worms moving in a corkscrew pattern instead of sinusoidally (Bergmann et al. 1998). While none of the known roller class of genes is present in this group, $hmr$-1 itself provides a further explanation of what may be occurring. $hmr$-1 encodes a classical cadherin required for proper hypodermal morphogenesis (Costa et al. 1998). As C. elegans muscle cells are directly attached to the hypodermis (Francis and Waterston 1991), hypodermal defects could result in muscle defects. In either case, be it the result of
hypodermal or cuticular defects, the muscle phenotype observed is most likely
not the result of muscle migration. Another point of note is, in a previous study
lev-10 was shown to be required in muscle for the proper clustering of
acetylcholine receptors at neuromuscular junctions (Gally et al. 2004). In that
study they did not report any defects in muscle positioning. So while it is possible
that proper enervation plays a role in muscle positioning, lev-10 may also
represent a false positive in the data set.

The other post-embryonic muscle phenotype was shared between let-805
and F56B3.2. In knockdowns for both these genes, worms were paralyzed and
10-20% of the paralyzed animals displayed gaps in their muscle quadrants (refer
to figure 4). let-805 encodes myotactin, a transmembrane protein that is involved
in the muscle-hypodermis linkage at the hypodermal side (Hresko et al. 1999).
As F56B3.2 displays a similar RNAi knockdown phenotype to let-805, it may also
be playing a role in muscle-hypodermis attachment.

The other RNAi phenotype of interest in my screen was embryonic
lethality. Of the nine genes that showed at least 20% embryonic lethality when
knocked down, six had been identified in previous mutational and large-scale
RNAi screens (refer to table 3) (Carter et al. 1990; Costa et al. 1998; Kamath et
al. 2003; Simmer et al. 2003; Sonnichsen et al. 2005). Looking at the distribution
of embryonic arrest phenotypes, three of genes, zyg-11, K07A12.2 and T09A5.9
were required in early embryogenesis either to maintain cell integrity, or for
proper cell division (data not shown). F33G12.4 knockdowns arrested before
gastrula formation and the remaining gene knockdowns arrested during
elongation (see figure 5). All tissues, including muscle, were disorganized in the regions of the defects, making it difficult to comment on the cause of the phenotype (data not shown). This illustrates the importance of using 4-D microscopy for this type of analysis.

4.2 Genes not identified in the screen

Muscle migration in *C. elegans* development is a well-defined event, but before my screen the genes regulating it were, and still mostly are, unknown. Looking through the genes that were identified, only laminin was found to be involved in muscle migration. The identification of F56B3.2 as possibly being a novel gene involved in muscle attachment was a bonus, but overall, a rather small group of genes showed any phenotype that could be construed as resulting from defective muscle migrations. The relatively low yield from the screen is, at least in part, probably due to poor penetrance of the RNAi. The variability of RNAi penetrance has been well documented in previous large-scale RNAi experiments, with the general consensus being that there is variability in the penetrance of RNAi knockdowns and that the phenotype exhibited by the knockdown does not necessarily represent the null phenotype of the gene (Maeda et al. 2001; Kamath et al. 2003; Simmer et al. 2003; Sonnichsen et al. 2005).

Both of these factors would have had a large effect on my screen. Not having a list of known muscle migration affecting genes makes it difficult to determine the efficacy of my screen, but we can look at the detection rate of other muscle affecting genes in my data set. For instance, RNAi phenotypes for
genes involved in muscle attachment (refer to figure 2) have been previously reported. These phenotypes vary from screen to screen and in some instances no phenotype for a gene was detected at all (Maeda et al. 2001; Kamath et al. 2003; Simmer et al. 2003; Sonnichsen et al. 2005). In this screen phenotypes were detected for pat-3, unc-52, let-805 and mup-4, while no phenotypes were detected for mua-3 or pat-2, giving a detection rate of ~66% (data not shown). The null phenotype for all these genes is embryonic arrest, but observed phenotypes in my screen ranged from larval arrest to paralyzed adults, consistent with previous screens. My ability to detect embryonic lethals was slightly worse. Eleven genes have been reported to result in embryonic lethality when knocked down by using RNAi feeding clones (Simmer et al. 2003; Sonnichsen et al. 2005) and I was able to detect 6 (~55%) of them. The caveat of using feeding clones is made here because, while it has been suggested that using feeding clones is as effective as introducing the dsRNA using microinjection (Kamath et al. 2001), using microinjection results in higher penetrance and earlier onset of RNAi phenotypes (Sonnichsen et al. 2005) (R. Viveiros personal observations).

Another difficulty in identifying muscle migration defects results from the likelihood of these proteins being involved in the migrations of different cell types at different life stages, specifically genes also required for distal tip cell migrations. C. elegans hermaphrodites have two U-shaped gonad arms and the distal tip cells are the cells responsible for proper migration of the developing gonad and defects in gonad arm development can lead to sterility (Lehmann
2001). As proof of principle, a previous large-scale RNAi screen for genes involved in distal tip cell migration identified a number of genes whose mutant null phenotype is embryonic arrest (Cram et al. 2006). Another example from this experiment is the laminin genes, as using RNAi feeding clones resulted in sterility of most P0s, but knockdown by microinjection caused embryonic lethality. In total 11 gene knockdowns (including those for epi-1 and lam-2) resulted in P0 sterility in this screen (Table 5) and are good candidates for follow up experiments to determine if they are involved in embryonic migrations as well.

One class of genes that I would have expected to find involved in muscle migrations is the cadherins. As mentioned previously, both M and N-cadherin have been found to play a role in vertebrate myoblast migrations (Cortes et al. 2003; Cinnamon et al. 2006). C. elegans has 10 genes that encode classical cadherins and three more that encode FAT-related cadherins (Hutter et al. 2000). Two of these genes, hmr-1 and cdh-3, have been characterized in detail and neither appears to have any role in muscle migration (Pettitt et al. 1996; Costa et al. 1998). Of the remaining genes, only 3 have any reported phenotypes, all from RNAi experiments; casy-1 has been reported to cause embryonic lethality when knocked using injection methods (Sonnichsen et al. 2005), though I have not been able to reproduce that result (data not shown), cdh-6 knockdowns using soaking methods result in sterility and cdh-1 displays a number of post-embryonic phenotypes when knocked down in a rrf-3 sensitized background (Maeda et al. 2001; Simmer et al. 2003). The rrf-3 RNAi sensitive strain is more susceptible to RNAi effects in the nervous system than wildtype, suggesting that
Table 5: Gene knockdowns causing Po sterility.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Concise Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0024.14</td>
<td>crm-1</td>
<td>Crim homolog</td>
</tr>
<tr>
<td>B0432.12</td>
<td>clec-117</td>
<td>C-type lectin</td>
</tr>
<tr>
<td>C37C3.6</td>
<td>ppn-1</td>
<td>Papilin homolog</td>
</tr>
<tr>
<td>C54D1.5</td>
<td>lam-2</td>
<td>Laminin gamma subunit</td>
</tr>
<tr>
<td>F15B9.7</td>
<td>cdh-6</td>
<td>Cadherin</td>
</tr>
<tr>
<td>F25H8.3</td>
<td>gon-1</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>F29G6.1</td>
<td></td>
<td>Possible protease inhibitor</td>
</tr>
<tr>
<td>K08C7.3</td>
<td>epi-1</td>
<td>Laminin alpha subunit</td>
</tr>
<tr>
<td>K08E5.3</td>
<td>mua-3</td>
<td>Transmembrane protein required for hypodermal attachment to the cuticle</td>
</tr>
<tr>
<td>R107.8</td>
<td>lin-12</td>
<td>Notch/LIN-12/glp-1 transmembrane receptor</td>
</tr>
<tr>
<td>T11F8.3</td>
<td>rme-2</td>
<td>Oocyte low-density lipoprotein receptor</td>
</tr>
</tbody>
</table>
cdh-1 may be playing a role there (Kamath et al. 2001; Timmons et al. 2001; Simmer et al. 2002). The lack of RNAi phenotypes for the other cadherin genes could indicate that there may be some redundancy in cadherin function in *C. elegans*, or that they are not very susceptible to RNAi knockdown in general. Performing RNAi screens targeting multiple cadherin genes simultaneously, or using one of the wildtype appearing cadherin mutants, may be useful in determining whether cadherins are involved in *C. elegans* muscle migration.

### 4.3 Identification of F33G12.4 as a novel lineage-affecting gene

*C. elegans* embryonic muscle arises from several distinct founding cell lineages. Of the 81 embryonically derived muscle cells, 32 are contributed from the C lineage, 28 from the MS lineage, 20 from the D, the only muscle exclusive cell lineage, and 1 from the AB lineage (Sulston et al. 1983). While they all ultimately generate muscle, the induction process varies between distinct cell lineages. Two maternal transcription factors, SKN-1 and PAL-1, appear to be the main regulators of embryonic myogenesis. PAL-1 is required to drive myogenesis in the C and D lineages, while SKN-1 is responsible for proper MS induction (Bowerman et al. 1992; Hunter and Kenyon 1996; Edgar et al. 2001). Ablation experiments have also illuminated a complicated interplay between different founder blastomere lineages, revealing that myogenesis is induced in both a cell autonomous and non-autonomous fashion (Schnabel 1995). SKN-1 and PAL-1 are both dependent on another transcription factor, POP-1, for the proper specification of myogenic fate. In the C lineage, PAL-1 in the presence low levels
of nuclear POP-1 specifies muscle fate, while in the presence of a high nuclear concentration of POP-1 a hypodermal fate is assigned (Lin et al. 1998). In contrast, in the EMS lineage, SKN-1 in the presence of high nuclear levels of POP-1 induces mesodermal cell fate, while in the presence of low concentrations of POP-1 the endodermal fate is assigned (Lin et al. 1998). In both cases the down-regulation of POP-1 is controlled through Wnt/MAP kinase signaling (Rocheleau et al. 1997; Thorpe et al. 1997; Meneghini et al. 1999).

F33G12.4 RNAi knockdowns display a loss of MS derived muscle cells as well as a transformation of the C hypodermal fate to muscle. Both of these phenotypes are consistent with a down-regulation of nuclear POP-1 (Lin et al. 1998). This could result in a couple of different ways: F33G12.4 could be involved in transcription of pop-1, it could be inhibiting the Wnt/MAP kinase signaling pathway, or it be could involved in the proper asymmetrical distribution of POP-1 during cell division, either specifically or through the general establishment of cell polarity. A scan of the available information reveals that F33G12.4 interacts with a dynein light chain in yeast two-hybrid experiments (wormbase website, www.wormbase.com, freeze ws170). As the dynein-dynactin complex is associated with generating asymmetrical cell divisions (Kaltschmidt and Brand 2002), F33G12.4 may be functioning in the same complex. If this is the case all asymmetrical cell divisions should be affected. Determining the subcellular localization of F33G12.4, or the distribution of POP-1 in the F33G12.4 knockdown embryos, would go a long way in elucidating how F33G12.4 is functioning.
4.4 Identification of laminin as a mediator of muscle migration

The laminin family of proteins is a key component of the ECM and it plays an important role throughout development. Five laminin alpha, three laminin beta and three laminin gamma subunits have been found in vertebrates and over 12 heterotrimeric isoforms are believed to be assembled (Burgeson et al. 1994; Iivanainen et al. 1995; Miner et al. 1995). Laminin appears to be the earliest basement membrane component formed during embryogenesis, with the expression of some subunits detectable as early as the eight cell stage (Cooper and MacQueen 1983). The proper secretion and assembly of laminin polymers is a crucial step in promoting further basement membrane assembly (Colognato et al. 1999; Li et al. 2003). That being the case, laminin plays an integral role in the formation of all basement membranes and unsurprisingly, a number of mutations in laminin subunits are associated with several human diseases, including congenital muscular dystrophy (Miner and Yurchenco 2004).

The *C. elegans* laminin family consists of two alpha, one beta and one gamma subunit, which together combine to produce two distinct heterotrimers (Hutter et al. 2000; Huang et al. 2003). Previous work has shown that laminin is required for embryonic viability, certain cell migrations, proper muscle attachment and polarity, and for proper tissue segregation (Huang et al. 2003; Kao et al. 2006). Work from this study has now shown that laminin is also required for proper dorsal and ventral muscle migration as well for the anterior migrations of the anterior-most ventral cells.
During embryogenesis transcripts for laminin subunits are first detected in the ingressing endodermal and mesodermal precursor cells at the onset of gastrulation. Expression later is restricted to pharyngeal, intestinal and myoblast cells. The laminin trimer is deposited between the rows of intestinal and pharyngeal precursor cells, the flanking myoblast cells, and the epidermal cells and is required to prevent the incorrect intermingling of cell types (Huang et al. 2003; Kao et al. 2006). What its role is in mediating muscle migration remains unclear.

One possibility might be that laminin is acting as a substrate for myoblast migration. Studies on cultured rodent myoblasts have shown that, in vitro, laminin can promote myoblast differentiation and migration and is dependent upon a muscle specific α7β1 integrin receptor (Foster et al. 1987; Ocalan et al. 1988; Goodman et al. 1989; Yao et al. 1996; Crawley et al. 1997). C. elegans has two α and one β integrins, INA-1, PAT-2 and PAT-3 respectively. Interestingly, the αPAT-2/βPAT-3 integrin receptor found in muscle cells appears to play no role in myoblast migration. Mutation of the integrin heterodimer found on muscle cells results in embryonic arrest at the two-fold stage of development, a time point after muscle cells have completed migration, and analysis of these mutants show no defects in muscle migration (Hresko et al. 1994; Gettner et al. 1995). While there is some evidence for the possible existence of another β integrin (discussed below), currently there is no indication that integrin is playing any role in muscle migration. Besides integrin, laminin can also be bound by dystroglycan (Colognato and Yurchenco 2000). Dystroglycan functions in muscle as part of the
dystrophin complex, and is important in muscle regeneration and defects in dystroglycan have been associated with muscular dystrophy (Cohn 2005). *C. elegans* has three genes that encode dystroglycan-like proteins, *dgn-1*, *dgn-2* and *dgn-3*, with *dgn-1* showing the highest similarity to vertebrate dystroglycan.

*dgn-1* is present in neurons and the epithelia, but not muscle. Null mutants are viable, but are sterile, due to gonad development defects (Johnson et al. 2006). Further characterization of the other dystroglycan-like proteins may yet reveal a role for it in muscle, but to date it, along with integrin, does not appear to be interacting with laminin during muscle migration.

As the known muscle receptors for laminin do not seem to be involved, the observed muscle migration defects may be the downstream result of other migratory defects. Laminin mutants affect a number of cell types and migratory defects in these tissues may result in incorrect cues for the migrating muscle cells. One candidate might be the hypodermis, as muscle migrations occur in orchestrated steps with myoblasts initiating at the hypodermal seam cell precursors and culminating with them laying beneath the dorsal and ventral hypodermal cells (Sulston et al. 1983; Hresko et al. 1994). Disrupting laminin does affect the basement membrane associated with hypodermal cells (Huang et al. 2003; Kao et al. 2006) and might result in defects that ultimately affect muscle. Further analysis of the hypodermal cells in the laminin mutants and knockdowns is needed to confirm this, but as ventral enclosure is occurring properly, any defects in the hypodermis cannot be too severe.
Interestingly, in the RNAi knockdown embryos only a subset of muscle migrations appears to be affected by the loss of laminin. Most cells along the elongated embryo reach their final destination, and while the ventral anterior-most cells fail to migrate properly, their dorsal counterparts invariably migrate correctly. Whether this observation is the result of the variability inherent to RNAi remains to be seen, as presently there are no null alleles for either the beta, or gamma laminin subunits.

4.5 Anterior muscle migration

One of the muscle migration defects observed in the laminin subunit RNAi knockdowns was a posterior displacement of the ventral anterior-most cells. This was unusual as previous studies have only described dorsal and ventral migrations (Sulston et al. 1983; Hresko et al. 1994; Moerman et al. 1996; Schnabel et al. 1997). In-depth 4-D analysis of wildtype embryos revealed that there are anterior muscle migrations occurring after the initial dorsal and ventral ones. Using the GFP labeled myoblast strain (PD7963) provided insight as to the mechanism behind these anterior migrations. As the muscle cells are migrating dorsally and ventrally to towards the hypodermal cells, the anterior muscle cells extend processes to the anterior. As the rest of the muscle cells reach they final destinations, these processes appear to serve as anchor points as the cell pulls itself to the anterior. The anterior migrations appear to be dependent upon these process extensions, since in the laminin knockdowns the posterior displaced, anterior-most ventral cells fail to extend them. Interestingly, in adults, knocking down laminin using RNAi results in the ectopic extension of muscle arms (Dixon
et al. 2006), not the loss of them as seen in this study. This may indicate that the role of laminin in muscle process extension may change during development, or that the loss of the embryonic muscle arms may not be a direct result of the laminin knockdown.

Previous studies that described C. elegans muscle migrations most likely missed this migratory event simply by chance. They happen late, in terms of muscle migration, and are fast. Looking at the muscle diagram from John Sulston’s seminal embryonic lineage paper, we clearly see that he stopped his analysis before the anterior migrations have happened (Sulston et al. 1983). This earlier work was also done without the benefit of 4-D microscopy, which greatly facilitates this type of analysis. The visualization of the anterior muscle cell processes also proved beneficial in elucidating what was happening and was only possible due to the GFP leaking out of the nucleus and labeling the cytosol. In previous studies using Nomarski optics or antibodies, it would have been very difficult to visualize the extending processes.

The discovery of anterior muscle cell migrations, and the processes extended from the anterior-most cells, begs the question: what proteins are involved in these events? Some answers can be gleaned from recent work from Min Han’s laboratory at the University of Colorado (U.S.). His group has been looking at a notched head phenotype that has been reported to be associated with certain mutant alleles of vab-1, vab-2 and ina-1, which code for an ephrin receptor, an ephrin and an alpha integrin, respectively (Baum and Garriga 1997; George et al. 1998; Chin-Sang et al. 1999). In their analysis they observed in
these notched head animals, the anterior-most ventral muscle cells are more posterior than in wildtype. Looking at the genes associated with the phenotype, both the ephrin and ephrin receptor are expressed primarily in neurons and have been implicated in proper hypodermal ventral enclosure (George et al. 1998; Chin-Sang et al. 1999). The alpha intergrin has been shown to be ubiquitously expressed during early embryogenesis and is required for proper morphogenesis of a number of tissues (Baum and Garriga 1997). Expressing the wildtype ina-1 gene in hypodermal cells is sufficient to rescue the notched head phenotype and results in wildtype muscle positioning (Min Han, personal communication). The discovery of laminin as also being required for these anterior migrations fits with these previous observations, as laminin has been well documented as being a ligand for integrin receptors (Belkin and Stepp 2000) and may be serving as a substrate for ina-1 mediated hypodermal migration. Determining whether the anterior-most ventral muscle cells also fail to extend in the ina-1, vab-1 and vab-2 mutants would lend further credence to this theory. An interesting aside from the above observations is, while ina-1 mutants result in defective head morphogenesis and displaced muscle cells, the same does not hold true for mutant alleles of pat-3, the beta integrin. As discussed previously, in pat-3 mutants, embryos arrest at the 2-fold stage of embryogenesis, but muscle cell migrations appear normal; the posterior displacement of the anterior-most ventral muscle cells seen in the ina-1 mutants does not occur (Hresko et al. 1994; Gettner et al. 1995). As PAT-3 is the only known beta integrin in C. elegans, both mutants should share similar phenotypes. Since this is not the case, there is a
strong probability of there being another unidentified beta integrin present in the genome.

In all, the discovery of anterior muscle migrations, and the extension of the muscle cell processes that appear to mediate them, provides an interesting, new area of study and a possible explanation for some previously observed phenomena. Further study will be required to tease apart the exact mechanisms mediating these migrations. A key question that remains is how do the dorsal anterior migrations differ from the ventral ones? All the identified anterior migration mutants only affect the ventral migration. Are the migratory signals different for dorsal and ventral anterior muscle cells, or is this just an effect of variable RNAi penetrance? While laminin does appear to be required for muscle migrations, other genes must be involved and remain to be identified.

4.6 Failure to identify genes involved in myoblast migration.

While the identification of laminin as being involved in myoblast migration is interesting, it represents the only protein identified in this screen. Many other genes must be involved and the question remains as to why this screen yielded so little. By choosing to focus on a subset of the genome from my screen, I limited the number of possible positives I could find. I still believe that focusing on the ECM proteins and their receptors was the correct path to take, but I could have broadened my data set to include other proteins, such as signaling molecules. I also focused only on the genes in my data set that had previously constructed feeding clones available (Kamath et al. 2003). This left nearly 200
genes that met my criteria, that I did not investigate, some of which may be involved in muscle migration.

RNAi may also not be the best method of disrupting ECM proteins. Looking at the 776 genes focused on in my screen, in all the previous large-scale RNAi screens combined, only 25 knockdowns resulted in embryonic lethality (Maeda et al. 2001; Kamath et al. 2003; Simmer et al. 2003; Sonnichsen et al. 2005) and some genes we know are required for embryonic viability, such as *emb-9* and *pat-2* show no embryonic lethality. RNAi may not be knocking down the message sufficiently, with enough product still being made to survive embryogenesis. Another possibility might be a high amount of genetic redundancy in myoblast migration. As discussed previously in regards to the cadherin family, the depletion of one protein is compensated by others in the process. To overcome this, knocking down multiple genes at the same time, or performing knockdowns in a mutant strain may be required.

All or none of these factors may have played a role in the low yield in my screen. In future experiments, I would ideally only use injection as an RNA delivery method and focus on genes previously shown to be required for embryonic viability or for post-embryonic cell migrations (Sonnichsen et al. 2005; Cram et al. 2006), as in these studies they did not investigate myoblast migrations. I would also like to investigate whether the cadherins play a role in *C. elegans* muscle migration, as they do in vertebrates (Cortes et al. 2003; Cinnamon et al. 2006). As a number of viable cadherin mutants have been
generated (C. elegans Knockout Consortium), they may be good candidates for future enhancer screens.
5 Conclusions

In an attempt to identify genes required for myoblast migration in *C. elegans*, I performed an RNAi based screen targeting genes predicted to be in the ECM or one of its receptors. Screening 776 genes that met these criteria resulted in the identification of one new gene, F56B3.2, as being possibly involved in mediating muscle attachment and the discovery of the requirement of laminin for proper muscle migrations. Analysis of the laminin knockdown phenotype has led to the identification of a previously uncharacterized anterior muscle migration that appears to be mediated by muscle arm extension. Both these anterior migrations, and the muscle processes that mediate them, provides a new phenotype to look for to help identify other genes involved in myoblast migration.
6 References


